Physiological Responses to Waterlogging in Barley (*Hordeum vulgare* L.)

by

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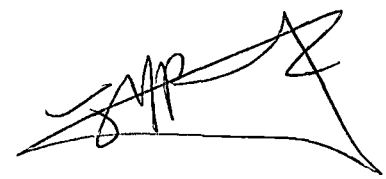
DECLARATION

The thesis contains no material, which has been accepted for the award of any other degree or diploma in any tertiary institution, and to the best of my knowledge, contains no material previously published or written by any other person, except where due reference is made in the text of this thesis.



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List of Abbreviations

ZP	Zhepi 1
DYSYH	Dongyang Sanyue Huang
TX9425	Taixing 9425
1(2,3)w	after 1(2,3) week(s) of waterlogging
1(2)wr	1(2) week(s) recovery following 3 weeks of waterlogging
IRGA	Infra Red Gas Analyzer
PAM	Pulse Amplitude Modulation
DW	dry weight
Pn	net photosynthetic rate
PSII	photosystem II
Fo	minimal fluorescence of dark adapted sample
Fv	variable fluorescence
Fm	Maximum fluorescence of dark adapted sample
LIX	liquid ion exchanger
KIR	potassium inward-rectifying channel
KOR	potassium outward-rectifying channel
NSCC	non-selective cation channel
SE	standard error
SEW	the sum of excess water
DACC	depolarization-activated Ca^{2+} channel

Abstract

Waterlogging is a major environmental constraint severely limiting crop production both in Australia and worldwide. In Australia, the problem is especially acute in higher rainfall areas with poorly draining soils. With most Australian commercial barley cultivars being waterlogging sensitive, waterlogging tolerant genetic material has to be selected and used in the breeding programs. Of specific interest is Chinese germplasm collection. However, lack of appropriate screening methodology makes selection of waterlogging tolerant cultivars difficult. Also, waterlogging is a complex abiotic stress encoding a large number of physiological and biochemical mechanisms and complicated by many confounding factors. Accordingly, the aims of this project were three-fold: (1) to develop an efficient screening methodology for waterlogging tolerance in barley breeding programs; (2) to select some waterlogging tolerant cultivars among existing barley germplasm; and (3) to understand the physiological, morphological and anatomical mechanisms encoding waterlogging tolerance in barley. The project was an integral part of GRDC-funded collaboration between Australia and China on barley genetics.

Barley plant growth was adversely affected by waterlogging. As waterlogging stress developed, chlorophyll content, net CO_2 assimilation and maximal photochemical efficiency of PSII (Fv/Fm) decreased significantly. Among these, chlorophyll fluorescence of dark-adapted samples (Fv/Fm values) was found to be the most efficient screening parameter for large-scale programs for waterlogging tolerance. Among studied genotypes, the Chinese cultivar TX9425 was found to be the most waterlogging tolerant, with the least reduction of plant growth, chlorophyll content, chlorophyll fluorescence and photosynthetic parameters. Naso Nijo (a Japanese cultivar) and Franklin (an Australian cultivar) were found to be the most susceptible to waterlogging. The adverse effects in stressed plants were alleviated following 2 weeks recovery in all cultivars. These contrasting genotypes were used later in all physiological studies.

The morphological and anatomical differences between two barley cultivars with contrasting waterlogging tolerance were investigated. In both cultivars, most seminal roots died under waterlogging conditions, while adventitious roots were produced at the shoot base. More adventitious roots were found in the waterlogging tolerant cultivar than in the susceptible cultivar. In adventitious roots of waterlogged plants, a substantial amount of aerenchyma was formed in the bulk of the root cortex except at the apex, facilitating O_2 transport from the aboveground parts into submerged roots. No aerenchyma was present in well-drained plants. The aerenchyma percentage along the whole root in the waterlogging tolerant cultivar was much larger than in the sensitive cultivar. The percentage of stele and xylem to the cross-section in adventitious roots was significantly reduced compared with the well-drained plants.

To understand the effects of waterlogging on nutrient acquisition and potential involvement of plasma membrane ion transporters in waterlogging tolerance in barley, the non-invasive microelectrode MIFE system was used to concurrently measure net O_2 and ion fluxes from the root surface. Oxygen deprivation caused the decline of O_2 uptake and an immediate and substantial effect on root ion flux patterns. These effects were different between waterlogging sensitive and tolerant cultivars. The O_2 uptake in the waterlogging tolerant cultivar remained much higher than in the waterlogging sensitive cultivar Naso Nijo in the root mature zone under hypoxia stress, while there was no significant difference in the root elongation zone between these two cultivars. In the mature zone, hypoxic treatment caused a very sharp decline in K^+ uptake in Naso Nijo, but did not reduce K^+ influx in the waterlogging tolerant TX9425 cultivar. In the elongation zone, onset of hypoxia enhanced K^+ uptake from roots of both cultivars. Hypoxia also caused qualitatively different effects on the activity of plasma membrane ion transporters in mature and elongation zones. Pharmacological experiments suggested that hypoxia-induced K^+ flux responses are likely to be mediated by both KIR and NSCC

channels in the elongation zone, while in the mature zone KOR channels are the key contributors.

Waterlogging stress is traditionally associated with O_2 depletion. Accordingly, breeding programs routinely target the plant's ability to tolerate O_2 deprivation and/or increase O_2 supply to roots. However, a significant accumulation of toxic substances from the microbial reduction processes has been widely reported in waterlogged soil. In this study, the effects of several secondary metabolites (phenolic acids, monocarboxylic acids and Mn^{2+}) on nutrient (K^+, H^+ and Ca^{2+}) acquisition of barley roots were investigated. All three lower monocarboxylic acids (formic, acetic and propionic acids) and three phenolic acids (benzoic, 2-hydroxybenzoic, 4-hydroxybenzoic acids) caused immediate net influx of H^+ and the reduction of K^+ uptake, while Mn^{2+} treatment caused K^+ to quickly return to the initial level following the net efflux in the first few minutes and gradual increase of H^+ influx. Phenolic acids slightly increased the influx of Ca^{2+} immediate after treatment, but not in other chemicals. Plant roots showed different responses of ion fluxes and membrane potential to these chemicals in the long term (24 h). 24 h treatment with all chemicals significantly reduced the K^+ uptake, and the adverse effects of phenolic acids were smaller than with monocarboxylic acids and Mn^{2+}. Treatment with monocarboxylic acids for 24 h reversed H^+ from net efflux to net influx, while all three phenolic acids did not cause significant effects compared with the control. Phenolic acids caused significant net Ca^{2+} efflux from roots pre-treated for 24 h. The possible model explaining effects of secondary metabolites on membrane transport activity is suggested.

In order to alleviate the adverse effects of waterlogging, the possibility of using foliar nutrient sprays was investigated. Foliar application of full strength Hoagland solution significantly improved plant growth, reduced leaf chlorosis and increased chlorophyll content, photochemical efficiency of PSII, net CO_2 assimilation, and production of adventitious roots. N and K content also increased, not only in shoots

but also roots, suggesting the translocation of nutrient from the shoot to root. This may be partially the reason for the greater production of adventitious roots in sprayed plants. Another contributing factor may be significantly higher amounts of auxin, accumulated in the shoot base of waterlogged plants (increased by 18.4%) after foliar nutrient application.

Overall, this study suggests that substantial genetic potential exists to improve waterlogging tolerance in barley. Measuring chlorophyll fluorescence of dark-adapted samples (Fv/Fm values) is recommended as an efficient screening tool for waterlogging tolerance in barley breeding program. Key features targeted by breeding should include both morphological (production of more adventitious roots and formation of larger aerenchyma area in adventitious roots) and physiological (high ability of O_2 uptake and K^+ acquisition in plant roots) traits, as well as the plant's ability to withstand soil-borne phytotoxins. Foliar application of nutrient can be used in practice to alleviate the adverse effects of waterlogging.

Chapter 1 General Introduction

1.1. Waterlogging as a world wide problem

Waterlogging significantly reduces growth and survival of plants, impacting agricultural production and causing enormous economic losses. It has increasingly becoming a matter of worldwide concern in many other agricultural areas (Ghassemi *et al.* 1995). It is estimated that around 10% of the global land area is affected by excessive water (Setter and Waters 2003). Annual production losses in Australia are around AU$180 million as a result of waterlogging (Price 1993). Waterlogging affects around 16% of soils in the USA (Boyer 1982). In northwest Europe, excess soil water, particularly in winter and spring, can also be an economic problem (Drew 1988). In south Asia waterlogging affects about 4.6 M ha, mainly in the irrigated areas in India and Pakistan (FAO online). Paddy soils occupy a large area of plains and polder lands along the Yangtze River Valley in China. Wheat and barley following flood irrigated rice can suffer in wet springs, due to poor soil physical structure. Long-term submergence under paddy rice results in a deterioration of root-zone characteristics (Lin *et al.* 2000).

Transient waterlogging occurs immediately after rain or irrigation when the soil physical properties prevent the water from draining freely. Permanent waterlogging occurs when a shallow water table restricts the free drainage of water from the root-zone of the soil. Some flooded or submerged soils can permanently occur, or are under water for at least several months every year. Paddy soils are the most well-known agricultural example of such soils. Both temporary and continuous flooding occurs in flood plains, marshes and irrigation areas throughout the world (Kozlowski 1984).

Waterlogging and flooding are not restricted to high-rainfall areas, as seasonal rains in semi-arid regions cause localized waterlogging (Rickman and Klepper 1980).

Poor drainage affects perhaps as much as one-half of the world's irrigated land (Kozlowski 1984), and flood irrigation when soil temperatures are high can rapidly deplete soil oxygen and damage crops (Drew 1988). In Australia, waterlogging occurs frequently in Australian dryland and irrigated soil. Transient waterlogging occurs primarily in sandy duplex soils, where rainfall rapidly penetrates a sandy topsoil and accumulates above a compacted clay subsoil with low hydraulic conductivity at 5->100 cm depth (Setter and Waters 2003, Tennant *et al.* 1992). The cost of drainage-related soil degradation to Australian agriculture is estimated at more than $270 million each year and the cost to growers can be as much as $138/ha in some years (CSIRO online). In Victoria, 3.8 million ha of duplex soils used for cereal production experience surface and subsurface waterlogging (MacEwan *et al.* 1992), and 60% of similar soils in Western Australia with yearly rainfall greater than 450 mm have widespread problems (MacFarlane and Cox 1992). In the Murray valley region of eastern Australia, a combination of salinity and waterlogging reduces total productivity by an estimated 16% (MacEwan *et al.* 1992).

Other causes of waterlogging are associated with rising groundwater and flooding in river basins (Grieve *et al.* 1986, McDonald and Gardner 1987). In recent years, the probability of flooding is greatly increased by human activities such as removal of natural vegetation, improvement of drainage systems further up the catchment, overgrazing by cattle, and straightening of meanders to facilitate shipping (Blom and Voesenek 1996). Precipitation reaches the stream system more directly and faster, often with a large load of sediment, enhancing the chance of flooding. To accommodate higher discharge, deepening and widening of the riverbed, as well as channeling, have been carried out in the upper reaches of many river systems. However, this causes increased downstream discharges and flooding.

1.2. Waterlogging stress and barley production

Barley (*Hordeum vulgare* L.) is one of the major world grain crops, with annual production of around 130 million tonnes being only exceeded by wheat, rice and maize (USDA online). It is widely reported that most commercial barley varieties are waterlogging sensitive. Cannell *et al.* (1984) found tillering in barley was reduced more than in wheat by identical waterlogged conditions. A combination of decreased tillers, seed numbers and seed weight resulted in a 30% reduction in final grain yield when barley was waterlogged with the water table 10 cm below the soil surface for 4 months (Cannell *et al.* 1984). Bandyopadhyah and Sen (1992) found barley was very sensitive to excess soil water, causing a loss in yield of 71 per cent when excess soil water persisted for two days, while it took 3 and 21 days for chili and cotton to lose more than 50%, respectively.

Most Australian commercial barley varieties are waterlogging sensitive, resulting in economic losses of millions of dollars. Among 7 major barley varieties in Australia, the grain yield of plants exposed to the intermittent waterlogging in the field was only 16-49% of non-waterlogged plants at $SEW_{30} = 40$ (equivalent to ~1 d WL to soil surface) (Setter and Waters 2003). SEW_{30} is the Sum of Excess Water that occurs each day in the primary root zone of the top 30 cm soil layer to quantify intermittent waterlogging throughout the year in the natural environment (Setter and Waters 2003). Units are centimetre days (cm d). However, the relatively higher yield of 2 varieties was a consequence of exceptionally high yields for non-waterlogged plants, instead of real waterlogging tolerance.

The above lack of waterlogging tolerant barley varieties is partially due to the lack of appropriate screening methodology, which makes selection of waterlogging tolerant genotypes difficult. Some genotypes may suffer from waterlogging but have good recovery and hence yield. Some very waterlogging tolerant varieties, on the other hand, may not be directly useful because of their slow growth and small

biomass. The problem is additionally complicated by the fact that waterlogging is a complex abiotic stress controlled by a large number of physiological and biochemical mechanisms and complicated by many confounding factors such as temperature, plant development stage, nutrient, and soil type. Accuracy of field-testing results can always be compromised because many cofactors fluctuate both spatially and temporally during the time-course of experiments in the field.

1.3. Objectives and research aims

There are three major objectives in this research:

(1) Develop an efficient screening methodology for waterlogging tolerance in barley breeding programs

The availability of efficient screening tools is crucially important to select potentially promising waterlogging tolerant genotypes in large populations in a barley breeding program. Conventional screening for plant tolerance to abiotic stresses in many breeding programs relies on visual observations in the field. This is a lengthy, labor- and time consuming, and rather inaccurate method. A technique that can give rapid, reliable, quantitative assessment of the injuries caused to plants by the stress factor is required. Therefore, the first objective of this research is to develop an efficient screening methodology for waterlogging tolerance in barley.

(2) Select some waterlogging tolerant cultivars within existing barley germplasm

As there is lack of waterlogging tolerant barley cultivars in Australia, waterlogging tolerant genetic material has to be selected and used in the breeding programs. Substantial genetic differences in tolerance to waterlogging have been found in barley. Setter *et al.* (1999) demonstrated a genetic diversity in waterlogging tolerance of barley exposed to intermittent waterlogging over 4 weeks. Of specific interest is the Chinese germplasm collection. This project is an integral part of

GRDC-funded collaboration between Australia and China on barley genetic resources. China holds more than 20,000 barley accessions, and barley is often grown in winter in rotation with rice as a summer crop, on soil prone to waterlogging during the barley-growing season. After a long period of evolution and selection, some varieties show better waterlogging tolerance and thus can be involved in breeding programs. So the second objective of this project is to select some waterlogging tolerant Chinese varieties and make them available to the Australian barley breeding program. It is recommended by Setter and Waters (2003) that to achieve waterlogging tolerance, an incremental process be followed by firstly incorporating adaptive traits from local, national or international germplasm with known tolerance, and then combining other adaptive traits relevant to the target environment.

(3) Understand the physiological, morphological and anatomical mechanisms controlling waterlogging tolerance in barley.

Different crops have developed different mechanisms to cope with abiotic stresses. Plants in waterlogged soil display a range of mechanisms to live temporarily without O_2. Metabolic acclimation can help plants to tolerate O_2 stress for a short time. However, for the long-term survival of plants in waterlogged soil, plants must be able to develop avoidance mechanisms. These adaptations are often displayed by shifts in anatomical and morphological characteristics. However, there are only very few studies on mechanisms encoding waterlogging tolerance in barley. Thus, it is important to understand the physiological, morphological and anatomical mechanisms encoding waterlogging tolerance in barley under both whole-plant and cellular levels.

1.4. Outline of the chapters

The thesis is divided into 8 chapters.

Chapter 1 is a general introduction in which the overall objectives are outlined.

Chapter 2 is a literature review on the topic of waterlogging and the reactions and adaptations to waterlogging stress.

Chapter 3 describes the general materials and methods used in this study.

Chapter 4 is an experiment chapter on the physiological responses to waterlogging and the following recovery among 6 barley cultivars. It aims to select some waterlogging tolerant cultivars and find a potentially efficient screening tool for waterlogging tolerance in barley.

Chapter 5 reports experiments on the immediate responses of O_2 and ion fluxes to hypoxia stress in barley roots. The responses in physiologically different root zones were compared and the involvement of ion transporters in the plasma membrane was also studied.

Chapter 6 characterizes the effects of secondary metabolites produced under waterlogging conditions on ion fluxes in barley roots.

Chapter 7 is another experimental chapter on the alleviation effects of foliar nutrient spray on waterlogging stress in barley.

Chapter 8 summarizes major findings reached in previous chapters and briefly discusses them in the context of the overall objectives. General conclusions are also presented.

Chapter 2 Literature Review

2.1. What happens in waterlogged soil?

2.1.1 Oxygen depletion

Deprivation of oxygen to the roots of plants is the main consequence of waterlogging. When the soil is waterlogged for long intervals of time and the pore space is completely filled with water, oxygen consumption by chemical and biological processes continues as long as some oxygen is available, but gaseous diffusion of oxygen into the profile is very slow (Gambrell *et al.* 1991). Water slows the diffusion of oxygen to 1/10,000 of that in air (Greenwood 1961). Within a few hours to a few days, depending on the energy source available for micro-organisms, soil oxygen levels can be depleted (Gambrell *et al.* 1991). Under normal conditions, water dissolves about 230 $mmol \cdot m^{-3}$ oxygen, and hypoxia occurs when the oxygen level falls below 50 $mmol \cdot m^{-3}$ (Grichko and Glick 2001). In some soil types, particularly sodic soils, the rapid addition of water can also result in a breakdown of soil structure, which reduces total pore space. The diameter and total volume of pores affects the amount of air in the soil as well as the rate of air diffusion and water movement into and out of the soil (Grieve *et al.* 1986).

2.1.2 Changes in redox potential and production of toxic substances

Excess water causes a sharp decrease in redox potential. The sequence of reduction takes place at specific redox potentials. As soon as free oxygen is depleted, nitrate is used by soil microorganisms as an alternative electron acceptor in respiration. Nitrate is reduced to nitrite (NO_2^-), various nitrous oxides (e.g. N_2O, NO), and molecular nitrogen (N_2) in the process of denitrification (Gambrell *et al.* 1991). This occurs at about 225 mV redox potential (corrected to pH 7) (Gambrell *et al.* 1991). Manganese oxides [mainly Mn(VI)] are the next electron acceptors when redox potential is at around 200 mV. In acid soils high in manganese oxides and organic

matter but low in nitrate, very high levels of water-soluble and exchangeable Mn^{2+} can build up within a few days (Marschner 1995). Ferric iron is reduced to the more soluble and mobile ferrous form just above 100 mV. Iron reduction is associated with a marked increase in soil pH (Kirk *et al.* 1990). Sulphate sulphur is reduced to the sulphide form at about –150 mV. The reduction of sulfate to H_2S in submerged soils may decrease the solubility of iron, zinc, copper, and cadmium by the formation of sparingly soluble sulfides (Ponnamperuma 1972). Finally, methane formation is initiated at around –200 mV from the reduction of carbon dioxide and certain organic acids (Gambrell *et al.* 1991).

Various products of microbial carbon metabolism, such as ethylene, also accumulate in waterlogged soils (Grichko and Glick 2001, Setter and Waters 2003). During prolonged waterlogging, volatile fatty acids and phenolics accumulate in soils high in readily decomposable organic matter (e.g., after application of green manure or straw), which has a detrimental effect on root metabolism and growth (Armstrong and Armstrong 1999, Lynch 1978, Tanaka *et al.* 1990, Wang *et al.* 1967).

2.2. Factors affecting waterlogging tolerance

2.2.1 Genetic variation in waterlogging tolerance in barley

Genetic variability of waterlogging tolerance was reported in barley. Screening of tolerance in barley germplasm was carried out on a large scale in China. 4,572 barley lines were tested by Qiu and Ke (1991) during 1986-1990 in Shanghai, China. A 'Damage index' was calculated by these authors as yield of plants in waterlogged conditions, expressed as a percentage of yield under non-waterlogged conditions. Waterlogging was imposed at three stages: leaf 3 stage, stem elongation stage and ear emergence stage for 10-15 days each. Varieties were classified into one of five grades of damage: 0.4% of varieties had <1% damage; 4.8% had 1-10% damage; 29.5% had 10-20% damage, 31.8% had 20-40% damage; the remaining

33.4% had >40% damage (Qiu and Ke 1991). Research by Ma and Gao (1990) in Zhejiang Province in China involved screening of 3064 barley varieties between 1986 and 1989. Waterlogging was imposed at stem elongation stage for 1 month. Calculation of damage index was based on plant height, number of green leaves, number of filled grains in each ear and weight of 1000 grains in waterlogging treatments, expressed as a reduction percentage of these indices under non-waterlogged conditions, with the sum of these percentage reductions as the general index. It was found that 6.89% of varieties had 40-60% damage, 7.25% of varieties had 60-80% damage and 85.87% had > 80% damage. Among Australian barley varieties, Setter *et al.* (1999) also found variability in waterlogging tolerance. Grain yield of 8 barley cultivars at SEW_{30}=160 cmd (equivalent to 5.3 d WL in the top 30cm of soil) was reduced by 51-84% of non-waterlogged.

It seems waterlogging tolerance is related to the origin and selection history of barley varieties. Research by Takeda and Fukuyama (1987) showed that among 3,457 barley varieties, varieties from China, Japan, Korea, and Nepal, as well as some varieties from North Africa, Ethiopia, and SW Asia showed good waterlogging tolerance at the germination stage. Many varieties from Western India showed the lowest tolerance. Ma and Gao (1990) found that there were more waterlogging tolerant barley varieties originating from the middle and lower reaches of the Yangtze River than from other areas in China. This might be because in these areas, barley is grown in winter in rotation with rice on soils very prone to waterlogging, and after a long period of evolution and selection, a large number of these barleys show better tolerance.

While the genetics of waterlogging tolerance has not been worked out, it has been shown that in general hulled barley was more tolerant than naked barley, and two-rowed types were more tolerant than multiple-rowed (Ma and Gao 1990, Qiu and Ke 1991). Hamachi *et al.* (1989) showed heterosis for tolerance of wet conditions, with the mean damage of F1 hybrids from 5 cross combinations in malting barley

under excess soil moisture treatment showing less damage than those of their midparents. Frequency distributions of the degree of dead leaf in F2 populations under excess soil moisture treatment showed continuous variation, suggesting polygenic inheritance.

2.2.2 Temperature

The rapidity with which waterlogging sensitive plants succumb is greatly dependent on the ambient temperature. The rate at which dissolved oxygen in the soil water is depleted is very sensitive to the soil temperature and the respiration rate of roots and micro-organisms (Drew 1983). With high temperatures and appreciable amounts of organic mater, or in soil mixed and incubated in the laboratory, depletion may be complete in only hours (Trought and Drew 1980a, Trought and Drew 1982). When temperatures are low and soil respiration is slowed, the concentration of oxygen in the water will decline only slowly (Trought and Drew 1982). Cai *et al.* (1994) found that high temperatures accentuate the effects of waterlogging and that there was a strong interaction between high temperature and waterlogging, which caused very significant decreases in number of green leaves on the main stem, chlorophyll content and ear moisture content. Trought and Drew (1982) showed that waterlogging damage was greater in plants at the higher soil temperature. Przywara and Stepniewski (1999) found that redox potential decreased under flooding conditions to 180, 150 and 70 mV at 7, 15 and 25 °C respectively, while it was maintained between 400 and 480 mV in the control plants.

2.2.3 Plant developmental stage

In rainfed or irrigated environments, waterlogging can happen at any plant developmental stage due to excess water. Leyshon and Sheard (1974) found that 14 day old barley plants at flooding were injured more than those which were 30 days old. However, the younger plants were more capable of recovery from the detrimental effects of flooding. Cannell *et al.* (1980) found that wheat was most

sensitive to waterlogging after germination but before shoot emergence. In their work, 16 days of waterlogging killed all seedlings and 6 days waterlogging depressed plant populations by 12-38% of that of the well-drained plants. When plants were waterlogged after emergence from the ground, the plant populations were not affected and there were little or no effects on grain yields in wheat (Cannell *et al.* 1980). Similar results were reported by Watson *et al.* (1976), where 6 weeks of continuous waterlogging in the very early growth stages of wheat, barley and oats resulted in greater reduction in root, herbage, and grain yield compared with waterlogging at the ear emergence stage. Results from Bao (1997) also showed that the responses of 20 wheat varieties and lines to waterlogging at different development stages were very significantly different. The order of susceptibility at different stages was booting stage > jointing stage > tillering stage > grain filling stage.

2.2.4 Time and duration of waterlogging

The highly variable nature of waterlogging in the field, in both space and time, emphasises the complexity of the problems of screening germplasm in the field. The Sum of Excess Water that occurs each day in the primary root zone of the top 30 cm soil layer (SEW_{30}) has been integrated and used by some researchers to quantify intermittent waterlogging throughout the year and at different soil depths in the natural environment (Setter and Waters 2003). Key features of SEW_{30} maps in duplex soils given by Setter *et al.* (1999) showed that waterlogging time and duration may vary by up to 400-fold over a distance of 50m, and considerable variation in waterlogging location and severity also occurred in different years at a specific site. Results from Malik *et al.* (2001) showed that plant growth was reduced proportionally as the water level was increased to the soil surface.

2.2.5 Soil physical properties

The soil physical properties affect the waterlogging severity on plants. Results of Cannell *et al.* (1984) showed that in the waterlogged treatment the oxygen concentration in the clay soil at all depths declined more rapidly than in the sandy loam to 2% or less. When the water tables were lowered, oxygen concentrations in the clay soil recovered more slowly than in the sandy loam, taking 2 weeks to return to atmospheric values at 20 and 50 cm depths. The effective duration of the anaerobic conditions was therefore longer in the clay than in the sandy loam (Cannell *et al.* 1984). The same features were shown in irrigated soils in NSW, where a shallow A horizon of loam to sand texture and a massive heavy clay B horizon gave severe waterlogging, whereas soils with a deeper A horizon of loam to sand texture have better drainage characteristics (Grieve *et al.* 1986).

The soil properties can therefore affect the growth response of plants upon waterlogging. Waterlogging-induced yield depression in wheat, barley and oats was less severe on upper than on middle or lower slope soils, part of which results from the more sandy nature of the upper slope soils (Watson *et al.* 1976). Waterlogging on a sandy loam had less effect on grain yield than waterlogging on a clay (Cannell *et al.* 1984). This might be partly explained by the more rapid rate of decline and slower recovery in the oxygen concentration in the clay, so that roots could be exposed to concentrations of oxygen limiting to root growth for a longer period than in the sandy loam. Also, there is increased likelihood of loss of nitrate by denitrification from the clay soil, which could have affected subsequent nutrient uptake and growth prior to nitrogen application in the spring (Cannell *et al.* 1984).

2.3. Physiological changes

2.3.1 Leaf chlorosis and death

Waterlogging affected barley plants typically show conspicuous chlorosis and early death of older leaves, as well as slower extension of leaves and shoots (Drew and

Sisworo 1979, Drew and Sisworo 1977). After 5 days waterlogging, the tips of the older leaves in barley became yellow and the yellowing gradually extended their entire length, while the younger leaves which developed and expanded during the course of flooding treatment contained a lower concentration of chlorophyll than controls (Drew and Sisworo 1977). Hamachi (1989, 1990) found that the degree of dead leaf under excess soil moisture treatment showed significant correlation with the reduction in culm length and grain yield per plant by waterlogging treatment in barley. He suggested that dead leaf percentage under excess soil moisture can be the best criterion for selection for flooding tolerance in early generations because its heritability values are relatively constant and it is easy to measure. However, there was no such correlation between leaf chlorosis and grain yields under waterlogged conditions in wheat, barley and oats exposed to intermittent waterlogging in Western Australia (Setter and Waters 2003).

2.3.2 Photosynthetic characteristics

Waterlogging induces functional disorders of the photosynthetic electron transport and CO_2 assimilation. Changes in photosynthesis, loss of photosynthetic capacity, degradation of the photosynthetic apparatus and accumulation of inactivated PSII centres can be partly understood as a process of adaptation to a decreased demand for assimilates (Drew 1983, Godde 1999). Slowed anaerobic carbohydrate catabolism leading to decreases in the rate of glycolysis may be due to its down-regulation or feedback inhibition (Gibbs and Greenway 2003). The prerequisites of anaerobic carbohydrate catabolism include the availability of substrate, and regeneration of the reduction of oxidised nucleotides (Gibbs and Greenway 2003). Feedback inhibition for accumulation of soluble carbohydrate can result in the reduction of the photosynthetic rate and cause the damage of the photosystem II reaction centre (Luxmoore and Stolzy 1969).

Prolonged flooding has been shown to have a significant impact on plant photosynthetic characteristics (Huang *et al.* 1997, Vu and Yelenosky 1991).

Significant reduction in net CO_2 assimilation and stomatal conductance in response to waterlogging was shown to occur in lupin (Davies *et al.* 2000), maize (Ashraf and Habib ur 1999), wheat (Huang *et al.* 1997, Huang *et al.* 1994b), rape (Zhou and Lin 1995), and some other crops. However, it is still unclear whether this effect is mediated by stomatal closure, or whether the leaf photochemistry is affected (Shabala 2002).

In recent years, chlorophyll fluorescence has been widely used as a tool in screening plants for response to environment stress. As a non-destructive method to monitor photosynthetic events at different functional levels from pigment level to enzymatic stroma reactions, it is very convenient to measure chlorophyll fluorescence (Maxwell and Johnson 2000). The number of publications on this subject has increased dramatically over the last few years. It has been used widely to assess the photochemical efficiency of photosystem II under various abiotic stresses such as salinity, drought, temperature, waterlogging, and nutrient disorders (Shabala 2002).

Studies involving thorough assessment of chlorophyll fluorescence characteristics in waterlogged plants are, however, rare. Wagner and Dreyer (1997) suggested that F_v/F_m ratio is sensitive enough to distinguish between waterlogging tolerance of different oak cultivars, but other authors believe that differences in root, but not leaf, functions are ones that should be used when selecting genotypes for enhanced waterlogging tolerance (Musgrave and Ding 1998). In normal, unstressed plants, this ratio Fv/Fm is usually very close to 0.83 for every species used (Bjorkman and Demmig 1987, Johnson *et al.* 1993). If the activity of PSII is affected by stress, the F_v/F_m ratio is significantly lower. A large drop in F_v/F_m ratio indicates a more severe effect of the stress factor (Shabala 2002).

2.3.3 Ionic relations in waterlogged plants

Plant mineral nutrition is a central aspect of the flooding response of plants. Ion transport in roots is highly sensitive to oxygen supply and marked changes in the

concentration of ions in the soil solution take place with flooding (Drew 1988). The link between oxygen supply and ion transport is mainly through respiration and the generation of ATP to drive transport. Anaerobic metabolism does not maintain energy metabolism at a level that will drive primary active transport via the H^+-translocating ATPase in the plasma membrane (Armstrong and Drew 2002).

Substantial reduction in ion uptake including N, P, and K in roots and transport to shoots in waterlogged soil and hypoxia solution has frequently been observed (Boem *et al.* 1996, Buwalda *et al.* 1988, Drew 1988, Singh *et al.* 2002, Trought and Drew 1980b, Trought and Drew 1980c). Drew and Sisworo (1977) observed a rapid decrease in nitrogen uptake and transport to the shoot in barley seedlings within 2 days with O_2 concentration in the soil water lower than 2%. Mg and Ca contents in the shoot were always less affected by oxygen deficiency than N, P, and K contents (Cannell *et al.* 1980, Drew and Sisworo 1979, Trought and Drew 1980a, Trought and Drew 1980b), suggesting that transport of these ions from the outer solution is less closely linked to energy metabolism (Stieger and Feller 1994).

The chlorosis and premature leaf senescence in flooded plants strongly resembles that of nitrogen deficiency. When young barley plants were made nitrogen deficient by briefly transferring them to aerated nutrient solution lacking nitrate, symptoms superficially indistinguishable from those of flooding were induced over a similar time scale, including leaf chlorosis, decreased N concentrations in the shoot, and a slower accumulation of dry matter by the shoot (Drew *et al.* 1979b).

Altered source-sink relations within the shoot can also influence the redistribution of phloem-mobile nutrients from older leaves to new growth (Boem *et al.* 1996). Oxygen deficiency in the root environment causes an earlier senescence of the oldest leaves. It was found that nitrogen compounds were rapidly remobilized within the barley plant, the older leaves losing their nitrogen to the younger leaves and tillers within 2 days, whereas leaves in the same position on aerated control plants continued a net accumulation of nitrogen (Drew *et al.* 1979b). An inadequate

delivery of nitrogen to the rapidly growing parts of the plant therefore promotes a redistribution of organic nitrogen compounds within the shoot and an abnormally early senescence of the older, N-exporting leaves (Drew 1988). The redistribution of nutrients within the shoot is not restricted to nitrogen; a redistribution of phosphorus and potassium also took place as an early response to waterlogging in barley (Drew and Sisworo 1979). In young winter wheat plants in waterlogged soil, although N, P, and K were translocated from the older leaves to the younger ones, they failed to completely compensate for the inhibited supply from the roots (Trought and Drew 1980b).

Significant increase in dissolved and exchangeable Fe and Mn in the waterlogged soil solution has been observed (Sharma and Swarup 1989, Stieger and Feller 1994). As a result, greater concentrations of Fe and Mn are usually found in the shoots of plants in flooded soil (Ashraf and Rehman 1999, Sharma and Swarup 1989, Stieger and Feller 1994) and unusually large accumulations of these metals are associated with toxicity symptoms (Drew 1988). The relative mobility ratio (RMR, the ratio of nutrient concentration in shoot to that in root for the particular nutrient) for Fe and Mn increases with increase in the duration of excess water conditions in soil, which indicates Fe and Mn contents in shoots increased at a relatively faster rate. The RMRs of Fe and Mn showed significant negative correlations with yield in barley, linseed, chili and cotton (Bandyopadhyay and Sen 1992).

2.3.4 Changes in hormonal status

Phytohormones act as intermediates between environmental signals and the plant's responses to these stimuli. The formation of adventitious roots (an important feature of waterlogging-tolerant plants) is regulated by hormones (Visser *et al.* 1996c, Wample and Reid 1979). In submerged plants, ethylene levels rapidly build up as the diffusion rate of this gas in water is approximately 10,000 times slower than in air and it is hardly metabolized in most tissues (Blom and Voesenek 1996). It was

widely recognized that ethylene promotes the formation of lysigenous aerenchyma which was formed by selective cell collapse in many plants (Brailsford *et al.* 1993, He *et al.* 1996a, Jackson 1994, McDonald and Visser 2003, Wample and Reid 1979). Ethylene action induces programmed cell death in files of cells of the cortex of primary roots in association with a disorientation of microtubules in cells destined to collapse, cell wall degeneration, and an increase in the activity of putative cell-wall degrading cellulases (Vartapetian and Jackson 1997). However, the formation of schizogenous aerenchyma, characteristic of many wetland plants which was formed by cell separation and differential rates of expansion is not under ethylene control as far as we know (Blom and Voesenek 1996). Ethylene levels exceeding 0.5 $\mu L\ L^{-1}$ are usually sufficient to evoke the maximum response in treated roots (Visser and Voesenek 2004). In non-aquatic plants, ethylene is often associated with shoot inhibition, leaf wilting and curling, all typical flooding responses. In contrast, ethylene is implicated in the promotion of shoot elongation in some semi-aquatic plants (Dat *et al.* 2004, Voesenek and Blom 1999).

Not only ethylene, but also other hormones are involved in the formation of flooding-induced adventitious roots. Due to high water levels, the transport of auxin from the shoot to the root is hampered which may result in accumulation of auxin at the shoot-root junction (Wample and Reid 1979). The higher concentration can trigger formation of adventitious roots. However, in *Rumex* species, differences in numbers of adventitious roots between flood-tolerant and flood-sensitive genotypes are not mainly due to higher auxin concentrations or to differences in sensitivity to auxin, but predominantly due to genetically determined capacities to develop this kind of roots (Visser *et al.* 1996b, Visser *et al.* 1995). The synergism between IAA and ethylene during adventitious root formation was reported by Visser *et al.* (1996c), where they found that high ethylene concentration sensitizes the root-forming tissue to auxin and the increased sensitivity to IAA subsequently induces the formation of adventitious roots in *Rumex* plants.

In addition, there is increasing interest in the role played by abscisic acid (ABA), gibberellic acid (GA), and cytokinin (Dat *et al.* 2004). Exogenous ABA applications caused increased anoxia tolerance in maize and Arabidopsis (Ellis *et al.* 1999, Hwang and Van Toai 1991). It is now believed that ABA action may be linked to its effect on GA. In rice, ABA was reported to be a potent inhibitor of GA action, and ethylene application reduces endogenous ABA levels (Hoffmann-Benning and Kende 1992). GA was also required for ethylene action in rice leaves during submergence (Raskin and Kende 1984). The synergism between GA and ethylene acts to increase the responsiveness of rice internodes to GA. It was found that the application of GA inhibitors inhibited ethylene and submergence-induced growth (Raskin and Kende 1984). Soil waterlogging inhibits synthesis of cytokinins by roots and reduces cytokinin fluxes in xylem sap of roots (Dat *et al.* 2004, Huang 1997). However, whether the reduction is due to lower biosynthesis or decreased transport from roots to shoots is still not demonstrated. It was found that exogenously applied cytokinins overcome the inhibition of shoot and stomatal conductance caused by perturbations of the root systems (Blackman and Davies 1983). Transgenic Arabidopsis plants with autoregulated cytokinin production were shown to be more waterlogging tolerant (Zhang *et al.* 2000).

2.4. Tolerance *vs* avoidance

It is likely that all higher plants can survive a certain period without oxygen, and phylogenetically there is no doubt that fermentation processes evolved before aerobic respiration (Armstrong *et al.* 1994a). Short-term or intermittent waterlogging primarily requires plants to maintain processes associated with survival, while growth is a secondary priority (Setter *et al.* 1999). Strategies that could be used include diverse traits such as the control of energy metabolism, the availability of extensive energy resources, the provision of essential gene products and synthesis of macromolecules, efficiency of nutrient uptake, and protection against post anoxic injury (Armstrong *et al.* 1994a). Tolerance to long-term

waterlogging requires plants not only to 'survive' but also to continue to grow during the waterlogging events. The key strategies used to overcome the effects of long term waterlogging are the exploitation of surface rooting, the development of aerenchyma in roots to facilitate gas diffusion and the rapid extension of various parts of the shoot system to establish gas-phase connection between the plant and atmosphere in response to submergence (Blom 1999, Jackson and Armstrong 1999).

2.5. Metabolic adaptations

Plant tissues use molecular oxygen for a number of biosynthetic or degradative processes, the major one being mitochondrial respiration (Ricard *et al.* 1994). The supply of oxygen to a tissue depends on its concentration and its diffusion rate in the surrounding medium. Hypoxia metabolism is characterized by both limited respiration (aerobic metabolism) and some degree of fermentation (anaerobic metabolism) (Ricard *et al.* 1994). Under hypoxic conditions, the metabolism of an organ may be heterogeneous since the outer layers receive more oxygen, and are therefore less hypoxic than the core of the tissue. Under natural conditions, anaerobic metabolism is very rare as traces of oxygen can still be detected.

2.5.1 Products of fermentation

Ethanol, lactate and alanine are the main products of fermentation in plant tissues. They all derive from pyruvate, the end-product of glycolysis. Ethanol is the major product of fermentation in higher plant tissues, whether they are tolerant to anoxia or not (Ricard *et al.* 1994). However, it is still not clear whether this fermentative pathway is functional under aerobic conditions, or at what level of hypoxia it becomes so. In higher plants, L-Lactic is often produced prior to ethanol in the first minutes after the transfer to anoxia (Roberts *et al.* 1984a, Roberts *et al.* 1984b). Lactic acid accumulation was considered to acidify the cytosol, thus providing the signal triggering ethanol production (Roberts *et al.* 1984a, Roberts *et al.* 1984b). Alanine is the third major fermentation product in plants (Reggiani *et al.* 1988).

Evidence of increasing levels of γ-aminobutyrate and succinate has been also confirmed in many different plants. In higher plants, the synthesis of succinate is a quantitatively minor pathway of anaerobic metabolism in terms of ATP production (Menegus *et al.* 1989), however, the operation of a partial tricarboxylic acid cycle may play an important qualitative role in providing precursors necessary for several biosynthetic pathways such as ammonium assimilation or heme synthesis (Ricard *et al.* 1994).

2.5.2 Control of energy metabolism

Under anoxic conditions when cytochrome oxidase activity becomes oxygen limited, ATP formation through oxidative phosphorylation is inhibited and ATP has to be produced by fermentation (Geigenberger 2003, Ricard *et al.* 1994). This impairs cellular metabolism and function because the efficiency of ATP formation is sharply reduced. The respiration of one molecule of hexose equivalent produces up to 39 molecules of ATP, whereas the fermentation of such a molecule provides a maximum of just three molecules of ATP (Beevers 1961). Many wetland species seem to possess a specialized metabolism that allows them to gain sufficient energy when there is not enough molecular oxygen to act as the terminal electron acceptor for the cytochromes (Armstrong and Drew 2002). The ATP required in anaerobic tissues is generated in glycolytic processes, mainly ethanolic and lactic acid fermentation (Armstrong *et al.* 1994a).

High concentration of sugars including fructans in roots during anoxia make starvation of respirable substrates seem unlikely, but transport of these sugars to the apical zone is not assured under anoxia (Bouny and Saglio 1996, Waters *et al.* 1991b). Root tissues are liable to lose reserves of sucrose quickly, because of inhibition of phloem transport to anoxic roots, where specifically the unloading step is affected (Waters *et al.* 1991a). With excised roots, exogenous supplies of glucose boost fermentation rates and energy metabolism and sometimes enhance survival of

intact roots or maintain normal mitochondrial structure, so that adequate provision of readily respired sugars may sometimes improve survival (Drew 1997).

2.5.3 Regulation of cytosolic pH

The regulation of cytosolic pH is considered to be the major determinant of plant tissue survival in anoxia. In maize or pea roots, cell death under anaerobic conditions is closely associated with acidification of the cytoplasm (Roberts *et al.* 1985, Roberts *et al.* 1984a). The glycolytic flux seems to be controlled by a pH-stat, starting with lactic acid fermentation. The pH of the cytoplasm (7.3-7.4 units) showed an early decrease that was attributed to an initial production of lactic acid (Roberts *et al.* 1984a, Roberts *et al.* 1984b, Roberts *et al.* 1992). Lactic acid accumulation was considered to acidify the cytosol, thus providing the signal triggering ethanol production. After about 20 min, the pH remained steady at 6.8, corresponding with a diversion of fermentation to ethanol. Lactate dehydrogenase (LDH) is inhibited by low pH, and fermentation soon switched to production of ethanol rather than to lactate as alcoholic dehydrogenase (ADH) enzymes work best below pH 7 (Armstrong *et al.* 1994a). Further acidification of the cytoplasm continued because of leakage of protons from the vacuole (Drew 1997). Proton-translocating ATPase in the tonoplast normally maintains this steep gradient, but with a decline in energy status its activity is presumed to be restricted, with passive leakage of protons to the cytoplasm (Armstrong and Drew 2002). Cytoplasmic acidosis is thus viewed as a determinant of cell death in plant cells (Drew 1997, Roberts *et al.* 1984a, Roberts *et al.* 1984b, Roberts *et al.* 1992, Xia and Roberts 1996).

It has been found, however, that lactate remains low in some cases such as rice seedlings (Menegus *et al.* 1991) or hypoxia-acclimated maize root tips (Xia and Roberts 1994, Xia and Saglio 1992), where ethanol production starts immediately. In a kinetic study of the changes of lactate, cytosolic pH and nucleotides, both after transfer to anoxia and following reoxygenation, it was found that cytosolic pH

changes much faster than the level of lactate, and closely follows the time course of the decrease of nucleotide triphosphate (Saintges *et al.* 1991). This suggests that the decrease in ATP is the main cause for the rapid acidification of the cytosol. Acidification may result from both inhibition of proton pumping at low ATP concentration and proton release through ATP hydrolysis. This would indicate that lactic acid is a minor component of the initial cytosolic acidification. However, its accumulation may still be responsible for the damage occurring under prolonged anoxia. The improved survival of normal maize compared with ADH deficient mutants has been related to the lower lactic acid production in the plant materials better able to withstand anoxia (Rivoal *et al.* 1991). It was found (Xia and Saglio 1992) that acclimated root tips not only produce less lactic acid, but also excrete it into the medium, thus resulting in higher cytosolic pH under anoxia than in the case of non-acclimated root tips.

2.5.4 Biochemical mechanisms of plant tolerance to oxygen deficiency

All plant cells are able to survive periods of anoxia of an hour or more and sometimes much longer, without cell death. Normally, the ATP content is sufficient for only 1-2 min in cells that are metabolically very active (Roberts *et al.* 1984a, Roberts *et al.* 1984b). Anaerobic metabolism must therefore contribute to cell survival in the short term by allowing ATP regeneration (Drew 1997). The biochemical basis for anoxia tolerance involves a combination of properties (Armstrong and Drew 2002). The essential features are the regeneration of nicotinamide adenine dinucleotide (NAD) from the reduced nicotinamide adenine dinucleotide (NADH) produced by dehydrogenases, a net synthesis of ATP (plant cell have no major store of ATP or other high-energy phosphate bonds such as pyrophosphates), and the production of end-products that either are compatible with metabolism or leak to the exterior where dilution renders them harmless. Additionally, dealing with metabolically generated protons is crucial (Armstrong

and Drew 2002). NAD is required only for the conversion of 3-phosphoglyceraldehyde to 1,3-diphosphoglycerate in glycolysis. This reaction is catalyzed by phosphoglyceraldehyde dehydrogenase; NAD is regenerated equally effectively in ethanolic fermentation or in conversion of pyruvate to lactic acid. The characteristics of anoxia-tolerant organs of wetland species include a sustained, predominately ethanolic fermentation, leakage of ethanol to the external medium or the transpiration stream, adequate reserves of carbohydrates to maintain glycolysis and energy metabolism, and in some cases high activity of starch phosphorylases (Drew 1997). Under anoxia, it is possible that pyrophosphate (PPi) might substitute for ATP as an energy source. Carystinos *et al.* (1995) showed that vacuolar H^+-pyrophosphatase (V-PPase) activity increased 75-fold after 6 days of anoxia in rice seedlings. Anoxia also induced an increase in activity of PPi: Fru-6-P 1 phosphotransferase, which substitutes for ATP-dependent phosphofructokinase in glycolysis in rice seedlings (Mertens *et al.* 1990).

Dealing with the accumulation of protons is particularly critical, because of the damage to metabolism that otherwise ensues (Armstrong and Drew 2002, Drew 1997). Formation of a variety of metabolites has been proposed as a means of consuming H^+ during anoxia, thereby offsetting cytoplasmic acidosis (Armstrong and Drew 2002). Succinate is one such metabolite. Menegus *et al.* (1989) found that anoxia-tolerant species tend to have higher ratios of succinate to lactate in the cell sap. Formation of γ-aminobutyric acid (GABA) from glutamate has also been suggested as a means of consuming protons and delaying cytoplasmic acidosis (Reid *et al.* 1985b). In maize roots, succinate production was negligible (Roberts *et al.* 1992) and most production of GABA occurred late in anoxia, when cells were close to death. However, decarboxylation of malic acid, through the low pH activation of malic enzyme, appeared to be consuming a significant quantity of protons during the early response to anoxia (Edwards *et al.* 1998).

2.5.5 Hypoxia pre-treatment

In nature, oxygen concentrations in waterlogged soil decline over periods of a few hours to several days (Trought and Drew 1982), depending on temperature, so that root cells gradually experience oxygen deficiency and they become transiently hypoxic before anoxic.

When intact maize or wheat seedlings are made hypoxic by subjecting them to a partial deficiency of oxygen (hypoxia pre-treatment), their subsequent ability to tolerate extended periods of anoxia is greatly improved (Saglio *et al.* 1988, Waters *et al.* 1991b, Xia and Roberts 1994). The improvement in anoxia tolerance was associated with an ability to maintain a greater rate of glycolysis and ethanolic fermentation as well as greater concentrations of ATP and total adenine nucleotides, relative to unacclimated root tips, in which respiration and energy metabolism collapsed after a few hours (Armstrong and Drew 2002). However, Xia *et al.* (1995) concluded that survival under anoxia is not closely dependent on the energy status of the cells. Hypoxically acclimated root tips survived anoxia and were better able to regulate cytoplasmic pH, whether or not ATP levels were depressed by metabolic inhibitors. Acclimation during hypoxia also involves an improved ability to transport lactic acid from the cytoplasm to the external medium. In hypoxically pretreated maize roots, anoxic stress caused less acidification of the cytoplasm and a greater transport of lactic acid to the exterior than in unacclimated roots (Xia and Roberts 1994, Xia and Roberts 1996, Xia *et al.* 1995).

2.6. Morphological and anatomical adaptations

Most flood-resistant plants are able to develop avoidance mechanisms to survive long-term waterlogging. These adaptations are based on rapid changes in physiological processes, often displayed by shifts in anatomical and morphological characteristics (Blom and Voesenek 1996). The anatomical basis of differences in

root oxygen transfer depends on species, on root type and even on the stage of development of individual roots.

2.6.1 Production of adventitious roots

Upon waterlogging, the initial effects in plants are in the root system. Developing adventitious roots is a mechanism to replace existing roots that have been killed or whose function is impaired due to oxygen deficiency (Vartapetian and Jackson 1997). Most wetland and amphibious species have roots containing highly porous tissues (Justin and Armstrong 1987), while the primary lateral roots of most terrestrial plants are not able to develop effective adaptations towards flooding. They succumb during hypoxia and new, adventitious roots will develop within a few days in flood-resistant plants (Blom and Voesenek 1996, Blom *et al.* 1994). Many non-wetland plants have a low gas-filled porosity when growing under adequate external aeration, but can produce roots with greater porosity when aeration is insufficient (Armstrong *et al.* 1991b, Visser *et al.* 2000). These roots usually grow from the base of the shoot, the hypocotyls and upper part of the tap root and on stem nodes, mostly exploring the upper better aerated soil layers (Blom and Voesenek 1996). It was also suggested that the ability to survive in anaerobic soils may be not due to a high resistance of root cells to anoxia, instead the roots of flooding tolerant species must gain access to a supply of oxygen and this is achieved by a re-directing root elongation either horizontally or upwards resulting in some roots reaching the water surface where oxygen is readily available (Vartapetian and Jackson 1997). The role of adventitious roots includes supplying water, minerals and hormones and acting as sinks for shoot assimilates and metabolites (Armstrong *et al.* 1994a).

2.6.2 Aerenchyma formation

For most species, the ability to withstand soil waterlogging is linked to internal transport of oxygen with sufficient capacity and speed required to avert root anoxia.

This is made possible by systems of internally interconnected gas-filled spaces, creating highly porous tissue known as aerenchyma, which has an inherently small resistance to the movement of gases (Jackson and Armstrong 1999). Aerenchyma provides a low-resistance internal pathway for the exchange of gases between the plant parts above the water and the submerged tissues; for example oxygen diffusion from the shoots to the roots and the venting of root- and soil-derived gases such as ethylene and methane from the roots to the atmosphere (Armstrong 1979). In well-adapted species, aerenchyma can extend from leaf stomata almost to the root tips and is capable of aerating roots up to 300-mm-long despite large losses en route from radial leakage and respiration (Justin and Armstrong 1987). In non-wetland plants such as wheat, however, it was found that adventitious root penetration was restricted to a depth of approximately 100 mm below the water level (Malik *et al.* 2001, Thomson *et al.* 1992).

Depending on the species and environmental conditions, aerenchyma can be formed in newly emerged adventitious roots, young seminal roots, the shoot base, and stems, petioles, and rhizomes (Armstrong *et al.* 1994a). The aerenchyma, however, usually terminates a few centimeters behind the tip in roots of wetland e.g. rice and non-wetland e.g. sunflower or wheat plants, so that O_2 diffusion from the aerenchyma to the apex may be enhanced in roots with cuboidal packing of cortical cells (Colmer 2003b).

2.6.2.1. Types of aerenchyma

Aerenchyma is formed either by selective cell collapse (lysigeny) or by cell separation and differential rates of expansion (schizogeny) (Justin and Armstrong 1987). Either "schizogenous" or "lysigenous" cells may predominate in different species (Justin and Armstrong 1987). The former is the outcome of highly regulated and species-specific patterns of cell separation and differential cell expansion that creates spaces between cells. Lysigenous aerenchyma arises from spatially selective death of grown cells (Jackson and Armstrong 1999). Lysigenous aerenchyma is

particularly common among the Poaceae and Cyperaceae, and schizogeny can be found in wetland plants such as *Caltha*, *Rumex* and *Filipendula* species (Armstrong *et al.* 1994a, Blom and Voesenek 1996). However, the two types can occur in the same plant. For example, lysigenous development may follow earlier schizogeny in the same organ (Drennan and Berjak 1982). Each kind can also form separately in different organs of the same species, e.g., lysigenous aerenchyma in the roots, and schizogenous aerenchyma in the leaves of *Sagittaria lancifolia* (Schussler and Longstreth 1996).

The type of aerenchyma formed can also influence the porosity just behind the root tip. Species with schizogenous aerenchyma, such as *R. palustris*, attain a high porosity within 0.5-1 cm of the root apex, whereas in roots with lysigenous aerenchyma the porosity increases for a considerable length behind the tip (2-6 cm). The functional significance of having one or the other type of aerenchyma has not been fully evaluated, but the larger volume of gas space closer to the tip of roots with schizogenous aerenchyma should enhance oxygen supply to the apex (Visser *et al.* 2000).

Schizogeny is a constitutive feature of many wetland species world wide, and the lacunae are created by a spatially regulated distribution of differential amounts of cell expansion and division, while lysigenous aerenchyma is the phenomenon of cell death (Jackson and Armstrong 1999). Lysigenous aerenchyma can readily be promoted by soil waterlogging or partial shoot submergence (Huang *et al.* 1994b, Visser *et al.* 1996a). The spaces formed are often separated by strands of surviving cells that are demonstrably alive as revealed by their ability to synthesise chlorophyll under illumination (Armstrong and Armstrong 1994). It is a prominent feature of some dominant wetland colonizers and of several major crop species such as rice, wheat, triticale, and barley (Jackson and Armstrong 1999). The cells that comprise these radial strands are self-evidently resistant to the cell death signals which kill their near neighbours.

2.6.2.2. *Arrangement of cells in the cortex*

Cell packing is mostly of two types: hexagonal, in which each cell makes contact with six near neighbours, and cubic, in which each cell contacts four near neighbours (Justin and Armstrong 1987). The most characteristic feature of hexagonal packing is close packing in outer cortical tissues, with extremely small to non-existent gas space (Justin and Armstrong 1987). Such tissues are detrimental at least in the young root in terms of aeration. Another characteristic of cells in hexagonal packing is that they seem to be less likely to subsequently form aerenchyma (Kawase 1981). Individual spaces are triquetrous in section and the maximum porosity with such an arrangement is only 9.3% (Armstrong *et al.* 1991b). The development of hexagonal cell packing may be generally regarded as being disadvantageous in terms of root aeration. The cubic packing of cells is a much better arrangement for aeration provision by internal gas transport than is hexagonal packing (Kawase 1981). The maximum achievable porosity is ~21.5% and cells in cubic packing generally form aerenchyma more readily in sub-apical regions (Armstrong *et al.* 1991b). Yamasaki (1952) calculated that the cubic type has 2.3 times as much air space per unit area of cortex, as the hexagonal type, if the cell sizes are equal. He found the cubic type to be more waterlogging-tolerant than the hexagonal type.

Hexagonal packing is chiefly located in root basal regions. The proportion of this outer cortical tissue diminishes and may ultimately disappear as roots extend into wetland sites (Justin and Armstrong 1987). It was found that in addition to cuboidal packing of the inner cortical cells, two wetland Cyperaceous species, and two wetland grass species *Oryza sativa* and *Echinochloa crus-galli* had cuboidal packing throughout the cortex. However, some dryland species and wetland species with inner cortex cuboidal packing had hexagonal packing in the mid and outer cortex (McDonald *et al.* 2002). These authors also found that extensive aerenchyma formation is not completely dependent on the cuboidal arrangement of the cortical

cells based on the discovery that some species with hexagonal arrangement of cortical cells had larger volumes of aerenchyma when grown in stagnant solution.

2.6.2.3. Importance of aerenchyma

Aerenchyma essentially serves two purposes (Armstrong *et al.* 1994a, Armstrong *et al.* 1991b), both of which facilitate gas transport: (i) it greatly reduces diffusive resistance to longitudinal transport of gases from shoot to root; and (ii) it causes a reduction in oxygen demand per unit volume in the organ in which it occurs. Of the two, it is the reduced diffusive resistance which accomplishes most in terms of elevated aeration. This was concluded by modelling studies in which the anatomical and respiratory demand characteristics of non-wetland and wetland root systems were intermixed (Armstrong 1979, Armstrong *et al.* 1991a). The effectiveness of internal oxygen transport in the plant depends chiefly upon the degree and distribution of two factors: the physical resistances to transport and the oxygen demands. Physical resistance is a function of plant root length, pore size, porosity, and path tortuosity (Jackson and Armstrong 1999).

Rogers and West (1993) found that rooting depths of a whole range of species increased with root porosity. The development of aerenchyma has also been shown to lead to higher energy charge and ATP levels in maize roots (Drew *et al.* 1985). It was reported that the variation in the % aerenchyma in the mid cortex of adventitious roots of wheat, barley, oats and triticale varieties was consistent with the general observation of the order of tolerance being oats and triticale > wheat > barley (Setter and Waters 2003). Positive correlations were also found between aerenchyma development and shoot growth or grain yield under hypoxia or waterlogged conditions with large numbers of wheat lines (Huang *et al.* 1994a, Setter and Waters 2003).

2.6.2.4. Enzymes related to aerenchyma formation

Lysogenic aerenchyma formation involves the death and often complete lysis of cells, with the disappearance of all cell components, including the cytoplasm and cell walls, so it is reasonable to assume that a wide array of cell-degrading enzymes are involved (He *et al.* 1996b). The programmed cell death in the cortex of maize roots is preceded by cell-wall degradation, which is linked to increases in the concentrations of cellulase (He *et al.* 1994, He *et al.* 1996a, He *et al.* 1996b). An increase in carboxymethylcellulase activity (as a marker for cell wall hydrolase activity) was also observed by Kawase (1979) in sunflower stem bases during flooding. He found cellulase applied to detached sunflower stem sections induces aerenchyma development comparable to that seen in waterlogged or ethylene-treated sunflowers (Kawase 1979). Cellulase also induces aerenchyma development in detached stems of bean, Brussels sprouts, cabbage, cauliflower, Chinese cabbage, chrysanthemum, corn, eggplant, and tomato plants (Kawase 1981). Interestingly, cellulase activity is increased by ethylene treatment in intact stems of bean, sunflower, and tomato plants (Kawase 1981, Kawase 1979).

Cellulase activity alone is not likely to cause cell enlargement and disintegration leading to aerenchyma and stem hypertrophy development, because not all cortical cells are affected evenly by cellulase application to sunflower stem sections (Kawase 1979). Disintegrating cells are plasmolyzed and the degree of plasmolysis is somewhat associated with the degree of cell disintegration (Kawase 1981).

Other enzymes are also involved in aerenchyma formation. Saab and Sachs (1996) detected transcripts of a gene with close sequence homology to a second putative cell-wall loosening enzyme xyloglucan endo-transglycosylase (XET) in roots and mesocotyl within 12 h of flooding maize seedlings. Expansins are a third group of enzymes with cell wall loosening properties. They work mainly through disrupting hydrogen bonding between load-bearing cellulose microfibrils or between microfibrils and their hemicellulose matrix (McQueen-Mason and Rochange 1999).

Certain expansins are thought to promote the action of hydrolases on cellulose by first detaching hemicellulose components (Jackson and Armstrong 1999)

2.6.3 Radial oxygen loss (ROL) from roots

In waterlogged soil, there are additional sinks for oxygen besides plant roots: the microbial and chemical oxygen demands, and these act effectively as competitors with the root for this internal oxygen supply. O_2 molecules within root aerenchyma will either be consumed by cells in adjacent tissues, diffuse towards the root apex, or diffuse radially to the rhizosphere and be consumed in the soil (Armstrong 1979). Studies showed that the rhizosphere does not just obtain oxygen which is surplus to the root's requirement (Armstrong and Beckett 1987). The flux of O_2 from the aerenchyma to the soil, which is termed radial O_2 loss (ROL), is determined by the concentration gradient, the physical resistance to O_2 diffusion in the radial direction, and consumption of O_2 by cells along this radial diffusion path (Armstrong and Beckett 1987). ROL can markedly reduce the supply of O_2 to the root apex (Armstrong 1979, Armstrong and Beckett 1987), with estimates up to 30-40% of the O_2 supplied via the root aerenchyma being lost to the soil (Armstrong 1979).

By means of radial oxygen loss to the rhizosphere, roots can affect sediment oxidation, protect the plant against the ingress of phytotoxins and support nitrification and other transformations as an aid to effluent purification.There is also evidence that radial oxygen loss from one species might enable other less well-adapted species to survive soil waterlogging (Callaway and King 1996), even though ROL reduces the supply of O_2 to distal root tissues (Armstrong 1979).

Models of the diffusive and respiratory characteristics of root and rhizosphere, which allow predictions of radial oxygen loss from root to soil, have been devised and are progressively being refined to describe more accurately what is an extremely complex system both spatially and temporally (Armstrong *et al.* 2000, Armstrong *et al.* 1994b, Beckett and Armstrong 1992). It is suggested that the

aerenchyma is essential to sustain the prolific lateral root growth which can be characteristic of some wetland plants, and it is these laterals which probably contribute most to sediment oxygenation and aerobic microbial activity (Jackson and Armstrong 1999).

2.6.4 ROL barrier

Many wetland species prevent excessive oxygen loss from the basal root zones by forming roots with a complete or partial 'barrier' to radial oxygen loss (ROL) in their epidermis, exodermis or subepidermal layers (Colmer 2003b, Jackson and Armstrong 1999, McDonald *et al.* 2002, Visser *et al.* 2000).

The role for O_2 leakage into the rhizosphere may appear contradictory to the notion that a barrier to ROL is an adaptive feature in roots of wetland plants. However, once the importance of spatial patterns of ROL within a whole root system are recognized (Colmer 2003b), these views are easily reconciled. Studies found that a barrier to ROL in basal root zones can enhance longitudinal O_2 diffusion in the aerenchyma towards the apex, by diminishing losses to the rhizosphere (Armstrong *et al.* 2000, Colmer 2003a, Visser *et al.* 2000), and also form numerous short laterals which emerge near the base of the main axes. The dense laterals with overlapping rhizospheres and high rates of ROL may enable an oxidized zone to persist even when O_2 is consumed by soil microorganisms. It is suggested by Armstrong (1979) that ROL may protect the apex and laterals from reduced soil toxins, whereas a physical barrier near the root exterior in mature zones presumably restricts entry for soil toxins in these regions. However, a physical barrier to ROL may also have drawbacks for some root functions. It was proposed that it may impede water and nutrient uptake by roots of wetland species (Armstrong 1979, Koncalova 1990).

In contrast to the many wetland species that have a 'tight' barrier to ROL, none of the dryland crop species examined to date such as wheat, rape, barley, rye, sorghum and oats had this feature (Colmer 2003b).

2.6.5 Leaf hyponasty and shoot elongation

It was found that in *Rumex palustris*, within a few hours of submergence, the orientation of rosette leaves changes from prostrate to almost hyponastic (upward) growth (Voesenek and Blom 1989). In addition, the youngest petioles increased their growth rate in the following several days. The elongation of shoot organs (coleoptiles, internodes, petioles, leaf blades, or flower stalks) in response to submergence was also observed in a broad range of plant species, mostly inhabiting aquatic or semiaquatic environments (Voesenek and Blom 1999). This enables the shoot to reach better illuminated and aerated zones close to the water surface, or preferentially above it (Armstrong *et al.* 1994a). Achieving this can not only capture more CO_2 and light by leaves above the water surface, but also can supply oxygen to the roots and vent potentially volatile phytotoxins from roots or other submerged organs (Voesenek and Blom 1999). In this way, the plant can resume aerobic metabolic activity required for long-term survival.

Submergence results in a fast increase in the endogenous ethylene concentration, with a 20-fold increase being observed within 1h in *R. palustris* (Banga *et al.* 1996). It was reported that the kinetics of ethylene accumulation matched the kinetics of the elongation response in *Regnellidium diphyllum*, deepwater rice and *Rumex palustris* (Voesenek and Blom 1999). The ethylene biosynthesis inhibitor strongly reduced ethylene production and the submergence-induced elongation of petioles (Voesenek and Blom 1989).

Ethylene is not the only plant hormone stimulating shoot elongation in submerged plants. Some aquatic and semi-aquatic plant species require auxin for maximal submergence-induced elongation (Voesenek and Blom 1999). In *R. palustris*, it was

found that auxin also plays an important role in submergence-induced petiole elongation (Cox *et al.* 2006). Gibberellin (GA) was found to be involved in the submergence-induced petiole elongation in *R. palustris* (Voesenek *et al.* 2003). It was also shown that ABA plays a role in the chain of events that leads to stimulated shoot elongation on submergence. ABA inhibits submergence-stimulated shoot elongation in deep-water rice, *Scirpus mucronatus*, and *R. palustris* (Voesenek *et al.* 2003).

2.7. Signalling and regulation of gene expression under O_2 deprivation

2.7.1 Changes in the pattern of gene expression

Sudden changes in oxygen partial pressure immediately lower the energy status of cells and give little opportunity for induction of an alternative metabolism. It was found that anoxic roots of maize displayed a remarkable change in the pattern of protein synthesis detected by PAGE (Polyacrylamide Gel Electrophoresis) with the overall protein synthesis reduced to 10-15% of the normoxic rate (Chang *et al.* 2000, Sachs *et al.* 1980). However, anoxia also stimulates the synthesis of a small group of proteins known as anaerobic ANP. The type and extent of ANP synthesis is directly related to the severity of oxygen deprivation, and to the plant species. About 20 anaerobic polypeptides (ANPs) were distinguished in maize, including ADH, pyruvate decarboxylase, glyceraldehyde-3-phosphate dehydrogenase, and other enzymes involved in glycolysis and fermentation (Dolferus *et al.* 2001, Sachs *et al.* 1980, Sachs *et al.* 1996). Proteomics identified a further 46 anaerobic proteins (Chang *et al.* 2000). Microarray studies have been used in recent years to identify transcripts and proteins that are involved in the low oxygen response in Arabidopsis and maize (Chang et al. 2000, Klok et al. 2002). Genes that are expressed differentially in response to low oxygen concentration encode not only well-known anaerobic proteins but also enzymes and signal-transduction components not previously known to be involved in low-oxygen metabolism (Geigenberger 2003).

Exposure of Arabidopsis roots to low oxygen caused both down- and up-regulation of a large number of genes. The up-regulated genes belong to three categories: (1) genes involved in ethanolic and lactic fermentation, (2) genes that potentially play a role in post-anoxic injury, and (3) genes related to ethylene synthesis, ethylene signalling, programmed cell death and cell-wall loosening (Klok *et al.* 2002). Dolferus *et al.* (2003) found that altogether 249 genes were significantly affected by the treatment of Arabidopsis roots for 20 h in a 0.5% oxygen. About 20% of the differentially expressed genes were down-regulated at the 0.5 h time point, but not all of these genes were repressed throughout the 20-h treatment period, and other genes were found to be down-regulated at other stages of the anaerobic response.

The alterations of gene expression occur at transcriptional, translational and post-translational levels (Klok *et al.* 2002, Sachs *et al.* 1980, Subbaiah and Sachs 2003). At the level of translation, anaerobic treatment of maize seedlings disrupts polysomes and leads to a redirection of protein synthesis (Subbaiah and Sachs 2003). However, the anaerobic messenger RNAs are not translated in direct proportion to their relative amounts. Profiling of the mRNAs in large polyribosome complexes confirmed that selective mRNA translation is a significant regulatory mechanism under hypoxia. The strongest impairment of protein synthesis is most probably a mechanism of energy conservation since a large proportion of cellular mRNAs show no reduction in steady-state abundance but are poorly translated under hypoxia (Bailey-Serres and Chang 2005). One example is sucrose synthase (SS1) in maize. Its transcript level is increased in maize roots without a concomitant increase in protein level (Taliercio and Chourey 1989).

Expression analysis reveals that increased transcription of genes is involved in the alcoholic and lactic fermentation pathways when external oxygen concentration is decreased to 5%, which can be interpreted as a pre-adaptation that allows continued energy production during a subsequent period of anoxia (Klok *et al.* 2002).

2.7.2 Signalling of waterlogging

Plants use external and internal signals to sense changes in the environment, such as shifts from aerial to aquatic (Visser and Voesenek 2004). Genes encoding the ANPs are rapidly turned on even by mild hypoxia and are rapidly turned off upon reoxygenation. Such a response implicates a fast and precise O_2 sensing system operating in plant cells. However, until now, the pathway leading to the perception and transduction of low O_2 signals remains unknown (Geigenberger 2003, Gibbs and Greenway 2003).

2.7.2.1. Sensing oxygen shortage

Oxygen levels in non-photosynthesising organs such as roots can decline rapidly due to continuous oxygen consumption in respiration, and the very slow delivery of aerial oxygen to the root. Falling oxygen levels are sensed in plants, and lead to a rapid down regulation of metabolism to decrease oxygen consumption, a shift to pathways that use oxygen more efficiently, and long term morphology changes to increase oxygen entry (Geigenberger 2003).

However, it remains unknown if the plant response is mediated exclusively by indirect sensing mechanisms or also involves direct oxygen sensing. An oxygen sensor in bacteria, yeast, insect and mammalian cells is capable of directly detecting oxygen availability and subsequently triggering a signalling cascade (Bailey-Serres and Chang 2005). It is still unclear if such sensors exist in plants. The plant oxygen-binding protein, haemoglobin, remains oxygenated at oxygen levels far below those that induce anaerobic responses (Dordas *et al.* 2003). This ruled out the possibility of haemoglobin as a potential oxygen sensor or carrier. However, it was found that in Arabidopsis one redox-sensitive transcription factor *ZAT12,* transcript levels were significantly elevated in response to hypoxia and anoxia, indicating involvement of this potentially redox-regulated factor (Branco-Price *et al.* 2005).

2.7.2.2. *Ionic homeostasis*

The sensing and signalling that lead to modification of gene expression may be triggered by a change in cellular homeostasis and not necessarily by a direct sensing of oxygen concentration (Bailey-Serres and Chang 2005). Deprivation of O_2 leads to disturbances in ionic balance of plant cells, reflecting energy depletion and membrane depolarization (Buwalda *et al.* 1988). Several studies showed that gene expression and physiological changes in response to O_2 deprivation are preceded and signalled by an elevation of cytosolic Ca^{2+} in maize seedlings and cultured cells (Subbaiah *et al.* 1994a, Subbaiah *et al.* 1994b). These authors found that ruthenium red (RR), a Ca^{2+} channel blocker, repressed the activation of the anoxia-inducible genes and impaired the post-anoxic survival of seedlings and cells. RR decreased the resting levels of $[Ca]_i$ and blocked the anoxia Ca^{2+} elevation. At the same time, caffeine, which induced an elevation of $[Ca]_i$ under aerobic conditions, caused an increase in ADH activity under normoxia (aerobic conditions) (Subbaiah *et al.* 1994a, Subbaiah *et al.* 1994b). It was found that the Ca^{2+} rise observed under anoxia was due to mobilization of the ion from intracellular stores. With the mitochondrion being the primary site of oxygen consumption and also an important target of RR action, it was thought it might serve as a Ca^{2+} store in responses to anoxia in maize cells (Subbaiah *et al.* 1994a, Subbaiah and Sachs 2003).

Under energy-deprived conditions, an imminent danger to cells is an unregulated traffic of ions. A continued elevation of Ca^{2+} or decline in pH is not only detrimental in the long run but may also impair the capacity of cells to mount the adaptive responses. Therefore, ionic homeostasis is probably a key component of the cellular adaptive pathway under stress. It was suggested $[Ca]_i$ changes could be important in the establishment of the pH-stat based on the discovery that plant glutamate decarboxylases (GADs) have a distinct ability to interact with calmodulin (CAM) (Shelp *et al.* 1999, Subbaiah and Sachs 2003).

So far, however, the idea of a Ca^{2+}-mediated signal transduction pathway mediating ethylene biosynthesis in maize roots exposed to oxygen deficiency has not been supported by experiment. Treatments with the Ca^{2+} chelator EGTA or a range of other substances that influence cytoplasmic Ca^{2+}, protein kinases or phosphatases, did not affect ethylene production when they were supplied topically to hypoxic roots (He *et al.* 1996b). The involvement of Ca^{2+} and animal-like signal transduction in the stimulation of ethylene biosynthesis induced by oxygen shortage remain unclear.

2.7.2.3. Ethylene perception

Ethylene biosynthesis increases within 4h of transfer to hypoxic conditions in several species (Drew *et al.* 1979a). Elevated ethylene levels are important for the induction of morphological and anatomical traits upon oxygen stress, such as production of adventitious roots and formation of aerenchyma. Progress in the understanding of ethylene perception and signal transduction in plants is improving very rapidly and is based on analyses of ethylene response mutants in Arabidopsis (Fluhr 1998). In brief, a family of at least five ethylene response genes (the *ETR1*-like genes) has been identified that code for ethylene receptor proteins. Closely related mRNA has been found in other species including *R. palustris* and maize (Vriezen *et al.* 1997) and its accumulation in leaves of *R. palustris* can be increased by submergence.

In plants, ethylene is perceived by a family of receptor molecules located in the endoplasmic reticulum (Chen *et al.* 2002). Ethylene receptors form a complex with a protein called Constitutive Triple Response (CTR) (Visser and Voesenek 2004). A notable achievement is the identification of *CTR1*, a gene that belongs to a family of serine/threonine protein kinases called Raf kinases. Raf kinases phosphorylate mitogen activated protein kinases (MAPKs) in signal transduction pathways that control growth and differentiation in animal cells. A similar role is hypothesised in plants. Like the ethylene receptors, *CTR1* is also a negative regulator, i.e., it

activates the downstream cascade by ceasing to phosphorylate when ethylene occupies the ETR receptor (Jackson and Armstrong 1999).

2.8. Conclusions

The responses of plants to waterlogging stress include physiological, morphological and biochemical adaptations on different levels: the whole plant level, tissue level, cellular level and molecular level. To identify genes regulating metabolic change, tolerance or avoidance of oxygen deficiency, should be useful for barley breeders to breed more tolerant crops. However, these cannot be easily measured. On the other hand, identification of associated physiological responses to waterlogging stress at the whole plant level and tissue level may lead to the identification and isolation of the genes conferring waterlogging tolerance. Thus the physiological researches in my study are important for barley breeders to breed more tolerant varieties.

Chapter 3. General Materials and Methods

This chapter covers general materials and methods used in the project, including those used in several experiments. Specific methods used in only one chapter will be dealt with in the appropriate place. A brief summary of the main experimental work done is listed in Table 3.1.

3.1. Plant material

Six barley cultivars (Naso Nijo, a cultivar of Japanese origin; Franklin and Gairdner, 2 Australian cultivars; ZP, TX9425 and DYSYH, cultivars of Chinese origin) were used in this research. The first 3 cultivars were supplied by the Tasmanian Institute of Agricultural Research (Launceston); ZP was obtained from Zhejiang University, China; and TX9425 and DYSYH were from Yangzhou University, China. All six are pure lines. Of these, Naso Nijo, Franklin, Gairdner, ZP and TX9425 are bred cultivars, whereas DYSYH is a local cultivar. TX9425 and DYSYH have mainly been used for animal feed, whereas the other four have been used essentially for malting purposes.

3.2. Growth conditions

3.2.1 Experiments in the glasshouse

Some experiments were carried out in a glasshouse with open slatted sides, providing substantial wind protection but maintaining temperature similar to that of the outside environment. Plants were grown under natural light during daytime, supplemented by 400W mercury lamps during morning and evening (PPFD 400 $\mu mol/m^2.s$; day/night 16/8 h). Daily irrigation was maintained by an automatic sprinkler system to provide optimal water supply. Plants were grown in 2-L pots filled with dark grey Vertosol soil, with 4 seedlings in each pot (Fig. 3.1). Dark grey Vertosol soil was collected from Cressy Research Station (Tasmania), a site where periodical waterlogging is a common problem, and used in most of the glasshouse experiments. Soil nutrient composition is shown in Table 3.2.

Table 3.1. List of main experiments

Experiment Title	Time	Design	Environment	Growing media
Growth and physiological responses of barley to waterlogging and subsequent recovery	Winter-Spring season in 2002	Split-plot design	Glasshouse, day/night temperatures between 13°C /10°C in August and 19°C /14°C in November.	Potting mixture
	Summer-Autumn in 2003-2004		Glasshouse, day/night temperatures between 21°C /14°C in December and 18°C /13°C in March	Dark grey Vertosol soil from Cressy Research Station (Tasmania)
Root anatomical study	Winter-Spring season in 2002		Glasshouse, day/night temperatures between 13°C /10°C in August and 19°C /14°C in November.	Potting mixture
O_2 and ion flux measurement in barley roots	2003-2004		In the lab, temperature +24°C; 16 h photoperiod; fluorescent lighting about 150 μmol m^{-2} s^{-1}	Aerated nutrient solution containing 0.1mM $CaCl_2$ + 0.2 mM KCl
Effects of secondary metabolites on ion fluxes in barley roots	2005			
Alleviation of foliar nutrient application on barley growth and physiological responses	Summer in 2004-2005	Split-plot design	Glasshouse, day/night temperatures varied between 22°C/14°C	Dark grey Vertosol soil from Cressy Research Station (Tasmania)
Hormonal experiment		Two-factor completely randomized block design		

Table 3.2. Some characteristics of Vertosol soil used for experiments

Parameter	*Vertosol soil*
N (total), %	0.72 ± 0.1
Exchangeable cations (cmol/kg)	
K	3.58 ± 0.18
Ca	16.49 ± 0.11
Mg	5.83 ± 0.06
Na	1.42 ± 0.02
Cu (mg/kg)	3.32 ± 0.16
Zn (mg/kg)	32.27 ± 0.23
Mn (mg/kg)	27.73 ± 0.11
Fe (mg/kg)	531 ± 7
Al (exchangeable) (cmol/kg)	0.015 ± 0.01
pH	5.55 ± 0.15

Fig. 3.1 Barley plants grown in pots in the glasshouse.

3.2.2 Hydroponic growth in Lab

Barley seeds were surface-sterilized by immersion in 1% NaOCl for 10 min and then washed thoroughly in flowing, distilled water for 5 min. The seeds were then put on a floating mesh in plastic containers above 0.5 L of aerated nutrient solution containing 0.1mM $CaCl_2$ and 0.2 mM KCl and grown hydroponically for 3 to 4 days under laboratory conditions (temperature + 24 °C; 16 h photoperiod;

fluorescent lighting about 150 μmol m^{-2} s^{-1}). Seedlings were used for measurement when their root length was 60 to 80 mm.

3.3. Waterlogging treatment

3.3.1 In the glasshouse

For waterlogging treatments, pots (2L with 12 cm diameter and 20 cm height) were placed in plastic tanks (75cm×30cm×25cm). The tank was filled with water and the water level was kept at the soil surface by an overflow hole on each tank at that height. Control tanks had holes in the bottom to allow drainage.

3.3.2 In the laboratory

A low concentration of agar solution was used to simulate the slow gas movements that occur in waterlogged soil (Wiengweera *et al.* 1997). Agar was dissolved in water first, then it was heated until the solution became completely clear, and cooled overnight to room temperature. Magnetic stirring was used until the temperature of the solution fell below 50 °C to prevent the formation of lumps. The agar concentration was 0.1% (w/v) in the final solution. Later $CaCl_2$ and KCl stock solution (100 mM) was added into it to make the final concentration of $CaCl_2$ and KCl become 0.1mM and 0.2 mM, respectively. 10 minutes before the hypoxia treatment in the laboratory, the 0.1% (w/v) agar solution was pre-bubbled with high purity N_2 (BOC Gases, 032G) to remove all O_2 from the solution. Then a certain volume of this agar solution was added into the chamber which already contains the same volume of normoxia solution, thus making O_2 concentration in the measuring chamber around 20 μmol L^{-1} to simulate waterlogging, resulting in a final concentration of 0.05% agar in the bath solution.

3.4. Whole-plant measurements

3.4.1 Growth measurement

Plants roots were washed carefully to remove soil and then harvested. Plant fresh weight was measured with a balance. Plant material was dried at 65°C in a Unitherm Drier (Birmingham, England) and then the shoot and root dry weights were determined.

3.4.2 Leaf chlorosis

Chlorotic leaves were scored visually, estimating the ratio of chlorotic area to total area in each leaf. The ratios for each leaf on the plant were summed to give a value for the number of chlorotic leaves in each plant.

3.4.3 Chlorophyll content

A fresh weight sample of ~0.1g was taken, from the youngest fully expanded barley leaf. Plant material was cut into small pieces, put in a vial, and 10 mL of 98% methanol was added as well as a small amount (~10mg) of $MgCO_3$ to neutralize organic acids. The samples were immediately placed in the dark and kept at 4°C for 48 h. A sub sample of 3mL of the extract was taken and put in a spectrometer cuvette. The absorbance was measured at wavelength 649 and 665 (OD_{649} and OD_{665}) respectively, using a Perkin Elmer UV/VIS Spectrometer (Lambda 20, Bodenseewerk, Ueberlingen, Germany), with 98% methanol as a blank control. Eight replicates for each of the cultivars and treatments were analysed and chlorophyll content was averaged over the eight replicates.

The following formulae were used to calculate chlorophyll *a* and *b* content (Smethurst and Shabala 2003):

$$\text{Chl } a \text{ (mg/L)} = 13.7\ OD_{665} - 5.76\ OD_{649}$$

Chl *b* (mg/L) = 25.8 OD_{649}-7.6 OD_{665}

Total chlorophyll content is expressed as the sum of chlorophyll *a* and *b*.

3.4.4 Chlorophyll fluorescence Fv/Fm

Chlorophyll fluorescence was measured *in vivo* from the upper surface of the youngest fully expanded barley leaves, using a pulse-amplitude modulation portable fluorometer (Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany). Barley plants were kept in the dark for at least 30 min in advance when measuring during the daytime. Plants can also be measured during the night. During the dark adaptation period, most electrons are drawn from Q_A (plastoquinone electron acceptor) leaving this pool in an oxidised state. Then a weak modulated light is given. As the intensity is very low (0.15 μmol quanta m^{-2} s^{-1}), it is insufficient to close light traps in the electron transport chain (ETC), but it is enough to establish a certain baseline for a constant fluorescence yield. As a result, chlorophyll fluorescence jumps from zero to a certain level denoted as F_0 (Fig. 3.2). This parameter reflects the emission from distal chlorophyll molecules and represents the light energy that was lost immediately as fluorescence and never reached the photosystem II (PSII) reaction centre. The next step is to saturate PSII with light. A brief (0.8 s) saturated light pulse of a high intensity (8,000 μmol quanta m^{-2} s^{-1}) is given, and Q_A acceptors start to saturate quickly. Once PSII absorbs light and Q_A has accepted an electron, it is not able to accept another until it has passed the first one onto a subsequent electron carrier (Q_B). During this period, the reaction center is said to be 'closed'. Due to temporary restrictions on the electron flow from Q_A to light harvesting complex (LHCII), a sharp rise in fluorescence emission from F_0 to F_m is observed (Fig. 3.2). At any point in time, the presence of a proportion of closed reaction centers leads to an overall reduction in the efficiency of photochemistry and thus to a corresponding increase in the yield of fluorescence.

Variation in strength of a fluorescent signal from F_0 to F_m is called the variable

fluorescence (F_v). The F_v/F_m ratio, measured after dark treatment, therefore reflects the proportion of efficiently working PSII units among the total PSII population. Hence, it is a measure of the photochemical efficiency of a leaf (Maxwell and Johnson 2000).

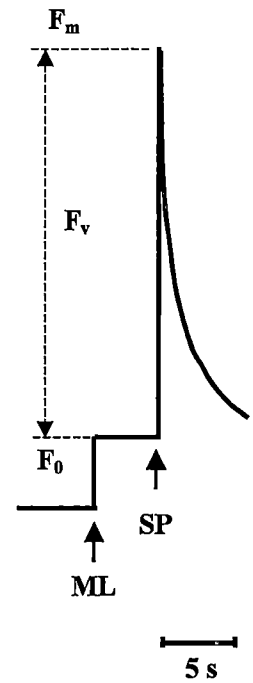

Fig. 3.2. Transient fluorescence kinetics and nomenclature of measured fluorescence characteristics. ML – measuring light; SP – saturating pulse. (Shabala and Pang 2006)

3.4.5 Photosynthesis, transpiration and stomatal conductance

Photosynthetic characteristics were measured with a portable infrared gas analyser (LCi-003 Analyser, ADC BioScientific Ltd, Hoddesdon, England). The main console of the instrument supplies air with a relatively stable CO_2 concentration to the leaf chamber at a measured rate. The CO_2 and H_2O concentrations are measured, and the air is directed over both surfaces of the leaf. The discharged air leaving the chamber is analysed and its CO_2 and H_2O content determined. From the difference in gas concentration and the airflow rate, the assimilation and

transpiration are calculated approximately every 20 seconds. A small fan in the chamber ensures thorough mixing of the air around the leaf. Measurement of CO_2 is by an infrared gas analyser. H_2O measurement is by two laser-trimmed humidity sensors. The reference CO_2 concentration was at about 370 ppm, H_2O reference was about 10 mBar, and the leaf temperature was around 23 ± 3 °C.

All measurements were made *in vivo* on the youngest fully expanded leaves in the glasshouse. The external photosynthetic light source (400W MF400 L/BU halogen lamp, G.E.C. Pty Ltd, Australia) was used to keep the light intensity constant at about 500 μmol m^{-2} s^{-1} during measurement. A plastic chamber containing cold water (functioning as an IR filter) was placed between plants and the external light source to prevent the over-heating of plant leaves.

3.5. Ion flux measurements

3.5.1 Non-invasive ion flux measurements: the theory

Ions can move either passively down their electrochemical gradient or against their electrochemical gradient, which is called active transport and requires energy. When an ion is taken up by living cell, its concentration in the proximity of the cell surface will be lower than that further away. *Vice versa*, if the ion is extruded across the plasma membrane, there will be a pronounced electrochemical potential gradient directed away from the cell or tissue surface. The principle of the MIFE (Microelectrode Ion Flux Estimation) technique is in measuring this electrochemical potential gradient by slow square-wave movement of ion-selective electrode probes between two positions, close to (position 1), and distant from (position 2) the sample surface (Fig. 3.3). At each position, electrode voltage is recorded and then converted into approximate concentration using the calibrated Nernst slope of the electrode. It is assumed that convection and water uptake are negligibly small and unstirred layer conditions are met (Newman 2001).

Net fluxes of specific ions (mol m^{-2} s^{-1}) can then be calculated from the measured voltage gradient near the surface. The magnitude of the flux is strongly dependent on the tissue geometry, determining ion diffusion profiles. In the simplest case of planar diffusion (such as from a plain leaf surface), the following equation is used (Newman 2001):

$$J = c\,u\,z\,F\,g\,(dV/dx),$$

where c is ion concentration (mol m^{-3}); u is the ion mobility (m s^{-1} per Newton mol^{-1}); z is the ion's valence; F is the Faraday number (96500 C mol^{-1}); g is a factor found from the measured Nernst slope for the electrode during calibration; dV is the voltage difference measured by the electrometer between the two positions (V); dx is the distance between two positions (m).

For cylindrical geometry (e.g. root surface) the radius of the cylinder (r) should be taken into account. This is done by replacing dx in the implementation of the equation above by

$$dx = r^2\,[1/(r+x) - 1/(r+x+dx)].$$

For spherical geometry (e.g. protoplast)

$$dx = r\,\ln[(r+x+dx)/(r+x)].$$

The theory of non-invasive MIFE ion flux measurements was reviewed in detail by Newman (2001).

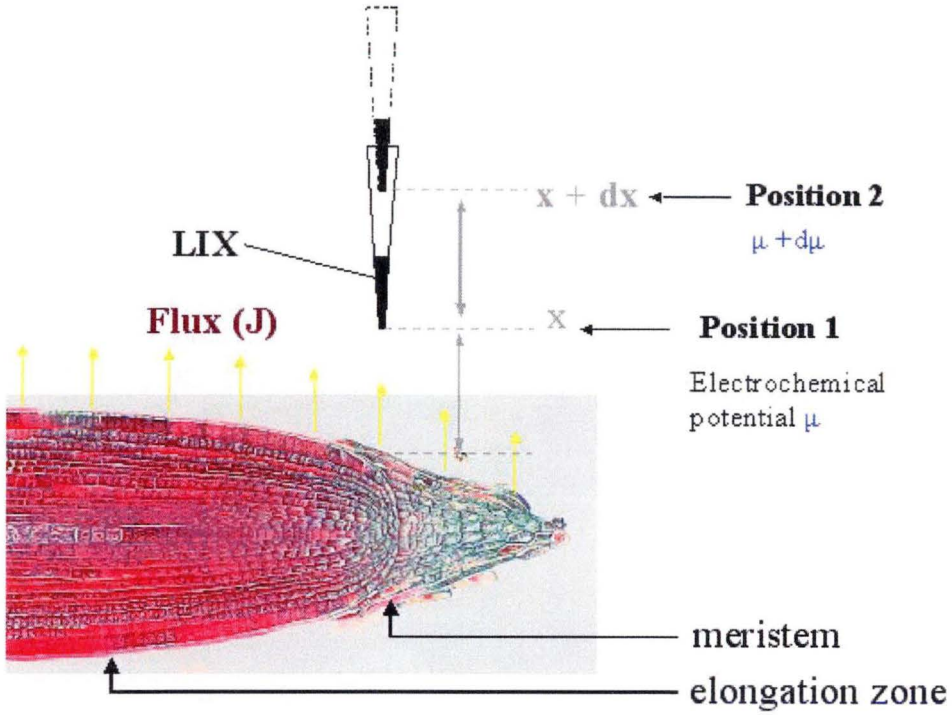

Fig. 3.3. Diagram illustrating the principles of MIFE theory. A microelectrode containing liquid ion exchanger (LIX) in the tip is moved vertically between two positions.

3.5.2 MIFE hardware and software

For ion flux measurements from a root surface, the MIFE setup is built around the standard stereomicroscope system with long distance objectives (200 x magnification). The ion-selective electrodes (up to three) are mounted on a MMT-5 multi-manipulator (Narishige, Tokyo, Japan) providing 3- dimensional positioning. This allows fine positioning of the electrode tips near the root surface. The measuring chamber is mounted on the computer-driven 3-dimensional hydraulics micromanipulator (Narishige WR-88). This enables the square-wave electrode movement to measure the electrochemical potential of the ions at two positions in solution close to a root surface.

The root tissue is immobilised at the bottom of the experimental chamber. The chamber is fixed on the microscope stand. The standard non-polarising Ag/AgCl reference electrode is positioned in the measuring chamber. The electrodes oscillate (usually at 0.1 Hz), between two positions, close and more distant from the roots, driven by the computer-controlled stepper motor. The voltage output from the electrodes is amplified by the MIFE electrometer and digitised using an analogue-to-digital interface card (DAS 08, Computer Boards Inc., USA) in a personal computer. The card also controls the stepper motor of the manipulator.

The DOS-operated CHART (University of Tasmania, Hobart, Australia) software package is used to control data acquisition by the MIFE hardware system. This software allows automated and interactive real-time control of the amplifier configuration and the micromanipulator while the data is being collected and written to disk. The system configuration is recorded together with the data, and all modifications during data acquisition are recorded in a log file which can also include annotations typed during the experiment. Flux calculations are performed automatically by the MIFEFLUX software (University of Tasmania, Hobart, Australia) from the data and log files. Calculated ion fluxes (in nmol m^{-2} s^{-1}) and concentrations are exported into an ASCII-format spreadsheet and saved onto computer disk, alongside the raw (mV records) data.

3.5.3 Microelectrode fabrication

Microelectrode fabrication includes several distinct steps: (i) pulling out electrode blanks; (ii) baking and silanising the blanks (making the electrode surface hydrophobic); (iii) backfilling silanised blanks; and (iv) front-filling electrode tips with appropriate liquid ion exchangers (LIXs).

The electrode blanks are made using 1.5 mm (OD) non-filamentous borosilicate glass capillaries (GC 150-10, CDR Clinical Technology, Middle Cove, Australia). The blanks are pulled to <1 μm diameter tips using a custom built vertical pipette

puller. Then electrode blanks are placed upright, base down, in a stainless steel rack and oven dried at 250 °C overnight. Ten to 15 min before silanisation, electrodes are covered by a steel lid that creates a closed container around the blanks, and 40-50 µL of tributylchlorosilane (90796, Fluka Chemicals, Busch, Switzerland) are injected under the lid. The lid is removed after 10 min and electrode blanks are baked at 250 °C for a further 30 min. By this procedure, the surface of the electrode blanks is made hydrophobic enabling entry of hydrophobic LIXs into the tip of the prepared microelectrode.

A LIX-containing tube is constructed using a glass microcapillary with ~ 30 to 50 µm tip diameter dipped into the stock LIX and thus containing a column of cocktail of approximately 1 mm long. The microelectrode blank is mounted horizontally on a three-dimensional micromanipulator and the electrode tips are flattened to achieve tip diameter of 2-3 µm by gently placing it against a flat glass surface while viewing under a stereomicroscope. Blanks with proper tip size are back filled with appropriate back-filling solutions using a syringe with a thin metal needle (MF34G-5, WPI, Sarasota, USA). Immediately after back-filling, the electrode tip is front-filled with the corresponding LIX. Once filled, the electrodes may last up to 24 h, if not longer, without significant changes in their characteristics.

3.5.4 Microelectrode calibration

Electrodes are fitted to the electrode holder and then calibrated against a set of three standards with a series of concentrations covering the expected range of the ion in question before and after use. Electrodes with responses less than 50 mV per decade for monovalent ions and 25 mV per decade for calcium, and with correlation coefficients less than 0.999 are discarded. Both the slope and intercept of the calibration line are used to calculate the concentrations of ions during the experiment. The resistance of the electrodes is typically 1-4 gOhm. The electrode resistance and the length of the LIX in the electrode tip are critical for electrode signal to noise resolution. High values are usually associated with increased 'noise'

of electrode response. The Ag/AgCl reference electrodes are fabricated in a similar way from a pulled and broken glass micro-capillary and filled with 1 M KCl in 2% agar. A chlorided silver wire (galvanised in 0.25 N HCl for 2-3 min) is inserted in a glass micro-capillary and sealed with parafilm. A small tip diameter (~50 μm) ensures little K^+ leakage from the reference electrode.

Chapter 4. Growth and physiological responses of six barley genotypes to waterlogging and subsequent recovery*

4.1. Abstract

In this study, the growth response of six barley genotypes of different origin (3 from China, 2 from Australia, 1 from Japan) to waterlogging and subsequent recovery was evaluated in two different soil types, an artificial potting mix and a Vertosol. A range of physiological measurements was assessed, to develop a method to aid selection for waterlogging tolerance. Plants at the 3 or 4 expanded leaf stages were subjected to waterlogging for 3 weeks followed by 2 weeks of recovery. Both shoot and root growth was negatively affected by waterlogging. As waterlogging stress developed, chlorophyll content, CO_2 assimilation rate, and maximal quantum efficiency of PSII (Fv/Fm) decreased significantly. The adverse effect of waterlogging was most severe for genotype Naso Nijo, intermediate for ZP, Gairdner, DYSYH and Franklin, and least for TX9425 in both trials. Studies of the root anatomy suggested that such a contrasting behaviour may be partially due to a significant difference in the pattern of aerenchyma formation in barley roots. The adverse effects in stressed plants were alleviated after two weeks of drainage for all genotypes. In general, TX9425 continued to grow better than other cultivars, while recovery of Naso Nijo was extremely slow. It is suggested that screening a small number of lines for waterlogging tolerance could be facilitated by selecting genotypes with least pronounced reduction of photosynthetic rate or total chlorophyll content, and for a larger number of lines, chlorophyll fluorescence is the most appropriate tool.

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4.2. Introduction

In Australia, transient waterlogging primarily occurs in sandy duplex soils, where rainfall rapidly penetrates a sandy topsoil and accumulates above the compacted clay subsoil with low hydraulic conductivity (Tennant *et al.* 1992). It is estimated that annual production losses in Australia are AU$180 million as a result of waterlogging (Price 1993). It is also increasingly becoming a matter of worldwide concern in many other agricultural areas (Ghassemi *et al.* 1995). Waterlogging affects around 16% of soils in the USA (Boyer 1982) and is also a major constraint on crop production in irrigated areas of India, Pakistan and China (Crosson and Anderson 1992).

Plant growth and physiological processes are adversely affected by soil waterlogging. It reduces shoot and root growth, dry matter accumulation and final yield (Drew 1991, Huang *et al.* 1994a, Huang *et al.* 1994b, Kozlowski 1984, Malik *et al.* 2001). For barley, waterlogging is estimated to reduce yields on average by 20-25%, but the loss may exceed 50% depending on the stage of plant development (Setter *et al.* 1999). Physiological consequences of waterlogging include decline in water uptake (Bradford and Hsiao 1982, Everard and Drew 1989, Reece and Riha 1991), reduced photosynthesis (Batzli and Dawson 1997, Bishnoi and Krishnamoorthy 1992, Singh *et al.* 1991) and altered root and shoot hormone relations (Jackson and Pearce 1991). Plants may also suffer from nutrient deficiencies due to reduced uptake of nutrients, denitrification and leaching of mobile nutrients, and the dilution of ions in waterlogged soil (Drew 1991, Trought and Drew 1980c).

Genetic differences in tolerance to waterlogging have been found in several species. Setter *et al.* (1999) demonstrated a genetic diversity in waterlogging tolerance of barley exposed to intermittent waterlogging over 4 weeks. Similar results have been also reported for many other plant species, such as *Zea mays* (Fausey *et al.* 1985,

Vantoai *et al.* 1988) and *Triticum aestivum* (Boru *et al.* 2001, Davies and Hillman 1988, Huang *et al.* 1994a, Huang *et al.* 1994b, Thomson *et al.* 1992). Waterlogging-tolerant plants may have specific morphological and anatomical characteristics enabling their survival and functioning under waterlogged conditions, such as aerenchyma formation and adventitious root development (Jackson and Drew 1984, Justin and Armstrong 1987, Laan *et al.* 1989).

Barley suffers yield reduction in waterlogged soils, but the amount of drainage work needed to alleviate waterlogging may not be justified by improved crop returns. Therefore the development of barley cultivars that tolerate waterlogging may be a sustainable solution for improving their productivity. It is necessary and important to develop techniques to screen for key aspects of waterlogging tolerance in barley, for breeders to improve this feature. China holds a substantial proportion of the world's barley germplasm in gene banks with more than 20,000 accessions, including 1500 wild barleys (Zhou and Mendham 2001). A large number of these barley genotypes has been selected by farmers for waterlogging, salinity and acid soil tolerance for centuries. Introducing Chinese germplasm into Australian barley breeding programs may be an important step in minimizing annual yield losses, especially for the cooler high rainfall areas of Australia.

One of the hurdles in selecting waterlogging tolerant cultivars is the lack of appropriate screening techniques. Another serious difficulty is the interpretation of the data, because genotypes tend to respond differently to waterlogging stress under varying experimental conditions. Obviously, the set of genotypes being compared, severity of the stress, stage of development of the crop and period of stress and, probably most important of all, the criterion chosen as the index of tolerance affects the ranking of particular genotypes in terms of tolerance to waterlogging stress (Akhtar *et al.* 2002). Earlier work in our laboratory showed that the use of the chlorophyll fluorescence technique (specifically, the Fv/Fm parameter) is a promising and reliable approach for quick screening of lucerne plants for

waterlogging tolerance (Smethurst and Shabala 2003). In the present study, I build on that work, extending the search for reliable methods of screening for waterlogging tolerant genotypes by conducting a comprehensive evaluation of the growth and physiological responses of six barley genotypes of different origin (3 from China, 2 from Australia, and 1 from Japan) to waterlogging and subsequent drainage.

4.3. Materials and Methods

4.3.1 Plant Material

Six barley cultivars (Naso Nijo, a cultivar of Japanese origin; Franklin and Gairdner, 2 Australian cultivars; ZP, TX9425 and DYSYH, cultivars of Chinese origin) were studied in this work. Details have been described in Section 3.1.

4.3.2 Growth Conditions

Experiments were carried out in pots with 2 different soil types in a glasshouse during the winter-spring season in 2002 and the summer-autumn in 2003/2004. In the first experiment (referred to below as Experiment 1), plants were grown in a mixture of sand: loam: sheep manure (2:1:1) in 2L black plastic pots of 12 cm diameter and 20 cm height. In the second experiment (Experiment 2), plants were grown in pots filled with dark grey Vertosol soil collected from Cressy Research Station (Tasmania), a site where periodical waterlogging is a common problem. The growth conditions e.g. temperature, light, have been described in Table 3.1. Soil nutrient composition for potting mixture is shown in Table 4.1 and for Vertosol soil is shown in Table 3.2. Each barley cultivar was planted into 28 pots (4 plants per pot). These pots were placed into black plastic tanks (2 pots per cultivar x 6 cultivars = 12 pots per tank; 14 tanks in total). Half of the tanks were used as controls, and the other half was subjected to waterlogging. A split plot design was used with tanks as main plots and cultivars as subplots. In total, each treatment by

cultivar combination had 56 plants; of these eight to 12 randomly chosen plants were analyzed as described in the next sections.

Forty days and 25 days after sowing when plants were at the four and three fully expanded leaf stage in the first and second experiment, respectively, half of the seedlings were subjected to waterlogging treatment for 3 weeks. The waterlogging treatment was described in Section 3.3.1. After 3 weeks of waterlogging, tanks were drained and maintained for 2 weeks while recovery was observed. Control tanks had holes in the bottom to allow drainage.

Table 4.1. Some characteristics of potting mixture used for experiments.

Parameter	*Potting mixture*
N (total), %	0.22 ± 0.2
Exchangeable cations (cmol/kg)	
K	3.20 ± 0.21
Ca	6.30 ± 0.48
Mg	3.04 ± 0.14
Na	1.68 ± 0.13
Cu (mg/kg)	1.63 ± 0.01
Zn (mg/kg)	28.08 ± 0.38
Mn (mg/kg)	16.26 ± 0.12
Fe (mg/kg)	128 ± 4
Al (exchangeable) (cmol/kg)	n.d.
pH	6.85 ± 0.05

n.d., not detected.

4.3.3 Chlorophyll content

Sequential extractions of chlorophyll *a* and *b* were made (after 1, 2, 3 weeks of waterlogging and the subsequent 1 and 2 weeks of recovery). The detailed procedures are described in Section 3.4.3.

4.3.4 Gas exchange (IRGA)

Gas exchange was measured with a LCI Portable Photosynthesis System Infrared Gas Analyzer (ADC BioScientific Ltd, Hoddesdon, England) on the youngest fully expanded leaves after 1, 2, 3 weeks of waterlogging and the subsequent recovery. Detailed procedures are described in Section 3.4.5.

4.3.5 Chlorophyll Fluorescence

Chlorophyll fluorescence was measured at a temperature of 20°C ± 3°C with a pulse-amplitude modulation portable fluorometer (Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany). Plants were kept in the dark for 30 min prior to measurements. Measurements were made in the middle of the youngest fully expanded leaf. Ten replicates of each cultivar/treatment were used. Details are described in Section 3.4.4.

4.3.6 Biomass

Twice during the experiment (after 3 weeks of waterlogging and then after 2 weeks of recovery from waterlogging), a sample of 8 each of the control and stressed plants was harvested for biomass analysis. Plant material was dried at 65°C in a Unitherm Drier (Birmingham, England) and then the shoot and root dry weights were determined.

4.3.7 Root Anatomy

Adventitious roots of one plant of the same genotype from each pot in the first experiment were collected for measurement, with five replicates of each genotype and treatment sampled. Freehand cross-sections of roots were cut every 2 cm, starting from 5 mm from the root tip back to the root-shoot junction, after vacuum-infiltrating the roots with water. Sections mounted on slides were stained with Toluidine blue O (1% w/v in 1% w/v borax solution) for 30 s and photographed under a light microscope for anatomical features. The area of aerenchyma in the root cortex, stele area, xylem area and total root cross-sectional area were determined with Scion Image analysis software (Scion Image program for windows Beta 4.02, US National Institutes of Health, Bethesda, MD, USA).

4.3.8 Data Analysis

The experiments ware carried out as randomised split-plot designs with each tank as a main plot, and the cultivars as subplots. Data for all growth and physiological parameters were analysed by analysis of variance (ANOVA) with the General Linear Model (GLM) using the Minitab Statistical Program (Minitab Release 13.2, Minitab Inc., USA). Differences between treatments for all measurements were compared using l.s.d. (P=0.05). The l.s.d. values for each figure are shown in Table 4.2.

Table 4.2. The l.s.d. (P=0.05) values for data included in figures.

Figure	*Parameter*		*1w*	*2w*	*3w*	*1wr*	*2wr*
Fig. 4.1A	SDW	Exp 1			0.17		0.36
Fig. 4.1B		Exp 2			0.07		0.25
Fig. 4.1A	RDW	Exp 1			0.03		0.04
Fig. 4.1B		Exp 2			0.03		0.07
Fig. 4.2A	Total Chl	Exp 1	0.17	0.16	0.18	0.16	0.17
Fig. 4.2B		Exp 2	0.15	0.13	0.12	0.15	0.16
Fig. 4.3A	Pn	Exp 1	2.01	1.95	1.56		
Fig. 4.3B		Exp 2	1.32	1.48	1.16	1.51	1.22
Fig. 4.4A	Fv/Fm	Exp 1	0.003	0.003	0.004	0.005	0.007
Fig. 4.4B		Exp 2	0.005	0.003	0.004	0.005	0.003
Fig. 4.7A	Longest root				8.58		
Fig. 4.7B	Adventitious root length				27.79		
Fig. 4.8	Aerenchyma				3.30		
	Stele				0.90		
	Xylem				0.004		

4.4. Results

4.4.1 Growth analysis

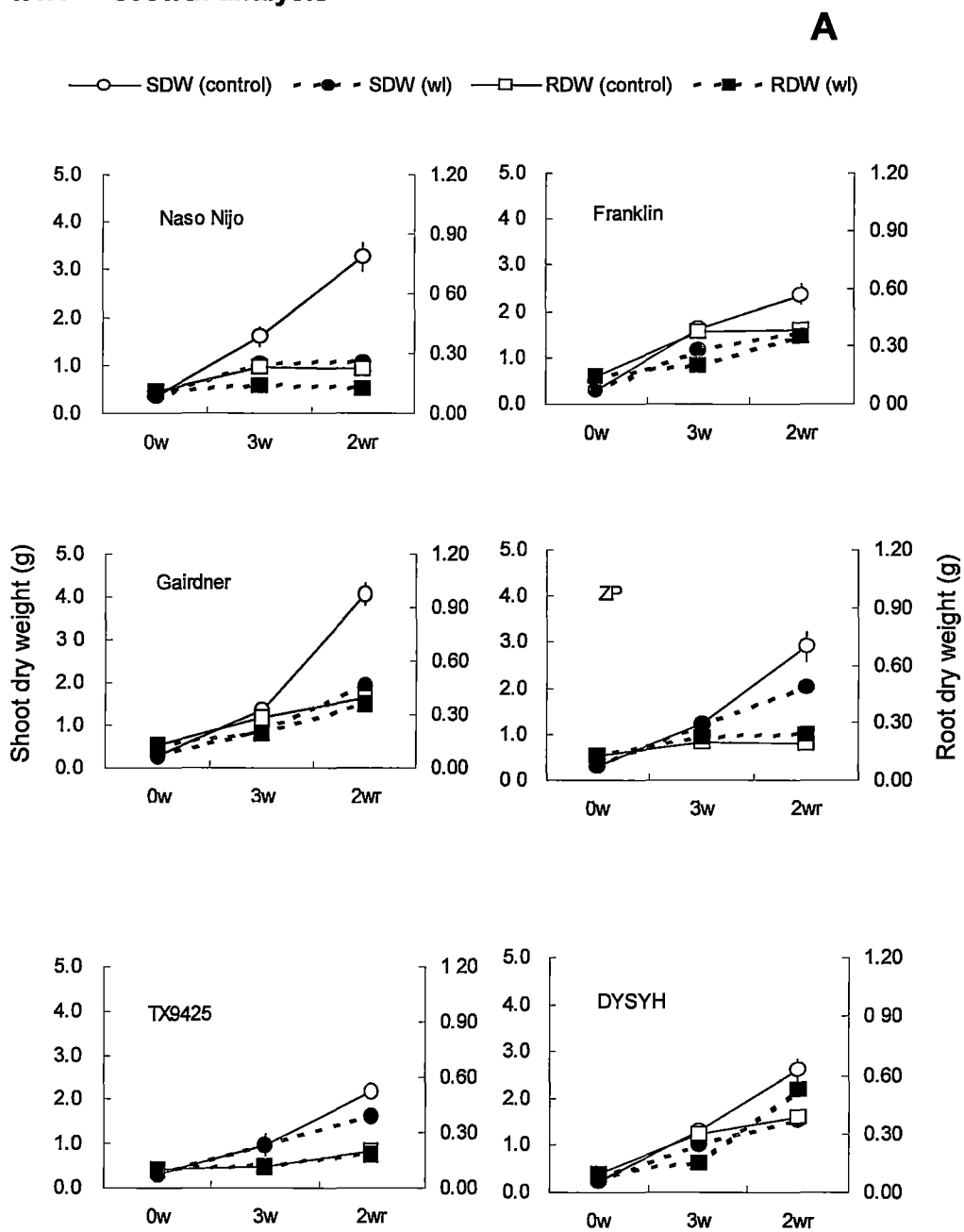

Fig. 4.1A Changes in shoot and root dry weight (SDW/RDW) for 6 different barley genotypes after 3 weeks waterlogging (3w) and 2 weeks of recovery (2wr), grown in artificial potting mixture. WL-waterlogging. Data are means ± SE (n=8).

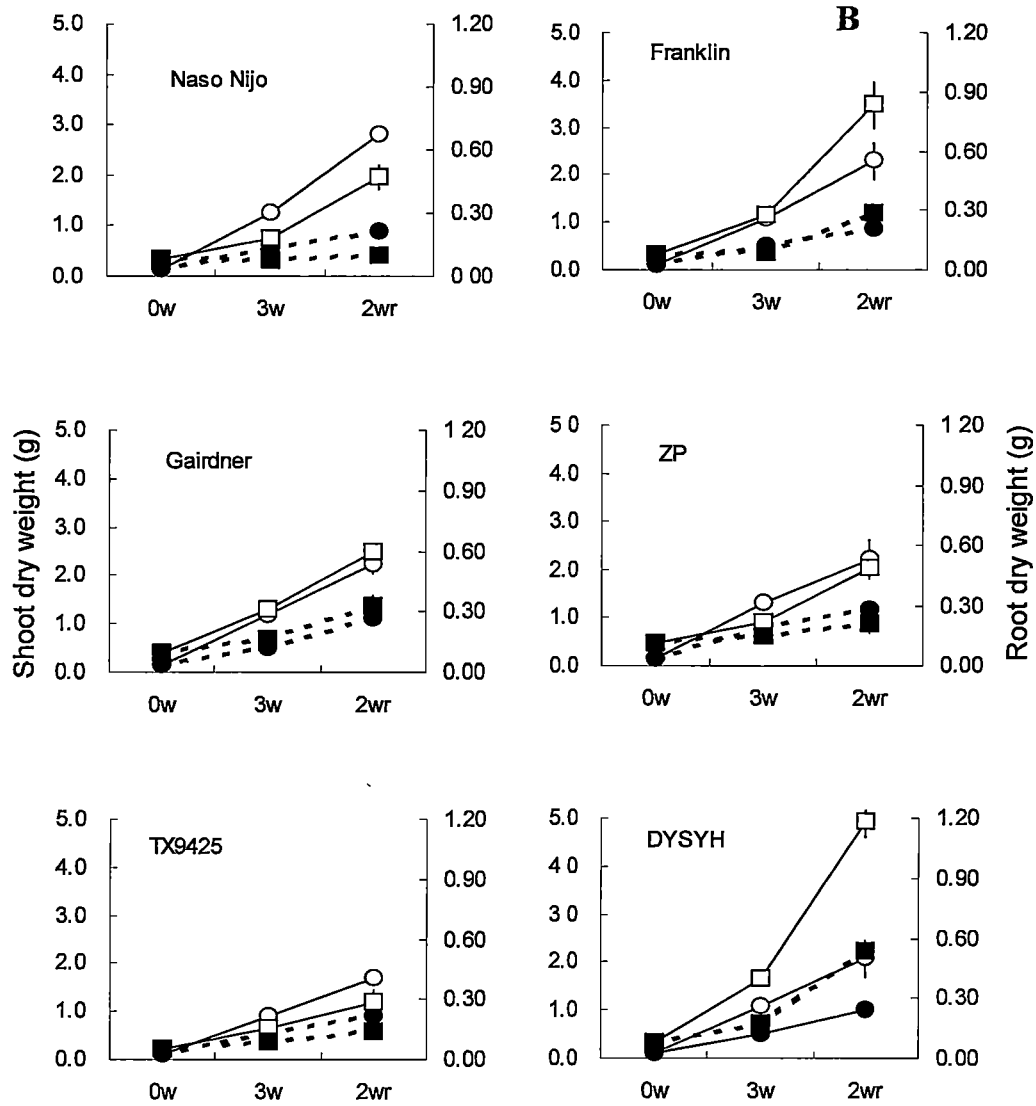

Fig. 4.1B Changes in shoot and root dry weight for 6 different barley genotypes after 3 weeks waterlogging (3w) and 2 weeks of recovery (2wr), grown in grey Vertosol soil. Data are means ± SE (n=8)

After 3 weeks of waterlogging, shoot dry weight (DW) of waterlogged plants in Experiment 1 was reduced to between 65% and 80% of the control for Naso Nijo, Franklin, Gairdner and DYSYH, with the corresponding figures for root DW between 50% and 70% (Fig. 4.1A). The shoot and root DW for TX9425 and ZP, however, did not decrease, and the root DW of ZP even increased slightly compared with the control. In Experiment 2, shoot DW of stressed plants was around 60% of

the controls for TX9425 and ZP, and root DW 56% and 67% respectively (Fig. 4.1B). The relative shoot DW in Naso Nijo, Franklin, Gairdner and DYSYH was between 43% and 52%, with the corresponding figures for root DW between 32% and 51%.

After 2 weeks of recovery, root DW in Experiment 1 was greater than the control in DYSYH and ZP, only slightly less than the control in Franklin, Gairdner and TX9425, but only 54% of the control for Naso Nijo (Fig. 4.1A). Shoot DW did not show complete recovery, being between 33% and 74% of control, smallest for Naso Nijo and largest for TX9425. In the more severe conditions of Experiment 2, root DW was within the range of 21-55% of the control, with Naso Nijo showing the lowest percentage, Gairdner and TX9425 the highest. In these conditions, shoot DW was 30-54% of control, also smallest for Naso Nijo and largest for TX9425 (Fig. 4.1B).

Regardless of the soil type, TX9425 and ZP showed better relative growth than other cultivars upon waterlogging and TX9425 also showed better relative growth after recovery, while Naso Nijo was the worst. For the absolute biomass of waterlogged plants, after 2 weeks of recovery following 3 weeks waterlogging, in both experiments ZP showed the highest absolute shoot biomass, the lowest was observed in Naso Nijo, also in Franklin in Experiment 2. DYSYH showed the highest absolute root DW and Naso Nijo showed the lowest in both experiments, while other cultivars were intermediate (Fig. 4.1A and B). In general, the adverse effects on shoot and growth in Experiment 2 (in "natural" soil type) were more severe than in Experiment 1 (in potting mix, an "artificial" soil substrate).

The number of tillers per seedling also decreased after 3 weeks of waterlogging in both experiments (data not shown). Expressing the waterlogged treatments as a proportion of the control, number of tillers per seedling in Experiment 1 was 60 ± 9% for both Naso Nijo and Franklin, 70 ± 15% for Gairdner and ZP, and about 80 ± 15% for TX9425 and DYSYH. After 2 weeks of drainage, the figure for Naso Nijo

was 47 ± 7% while there was no significant difference among the other five genotypes, ranging between 63% and 72%.

4.4.2 Chlorophyll Content

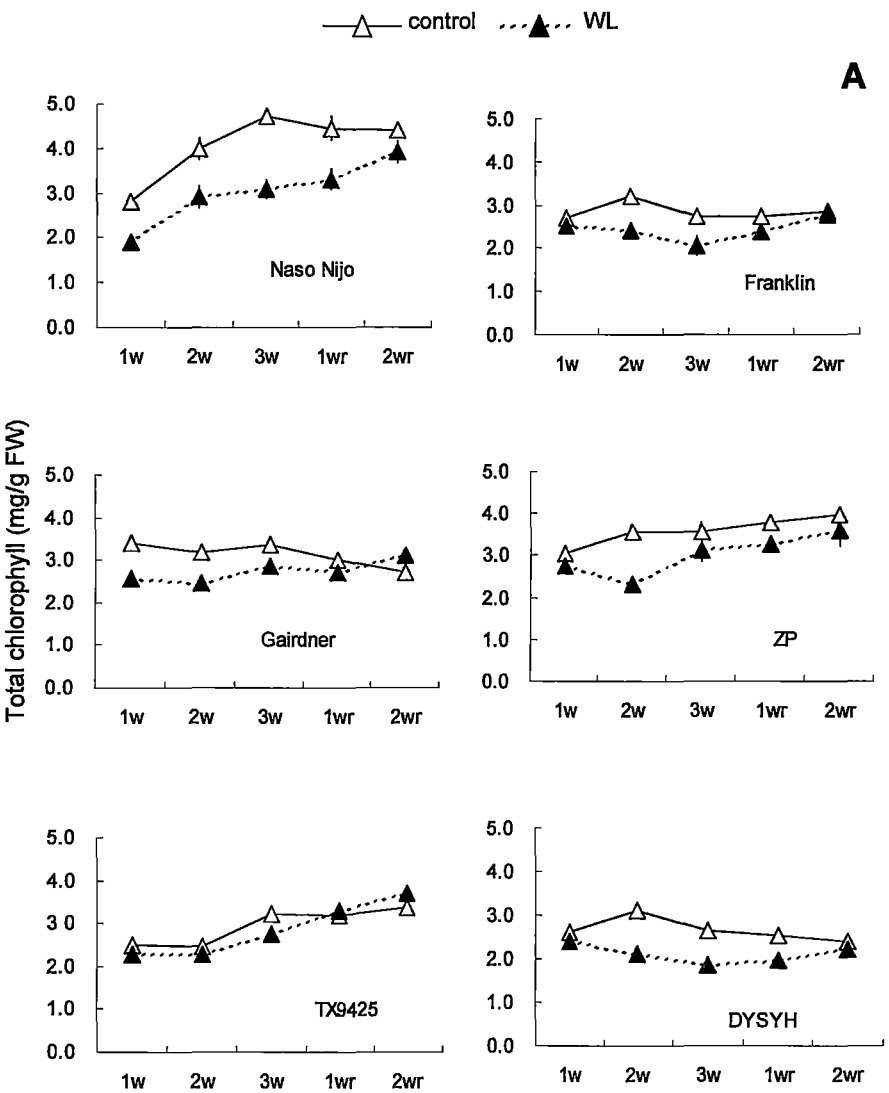

Fig. 4.2A Changes in leaf pigment composition in response to waterlogging (WL) and subsequent drainage in 6 barley genotypes grown in artificial potting mixture. Data are means ± SE (n=8). Note that in most cases, the error bars are smaller than the symbols.

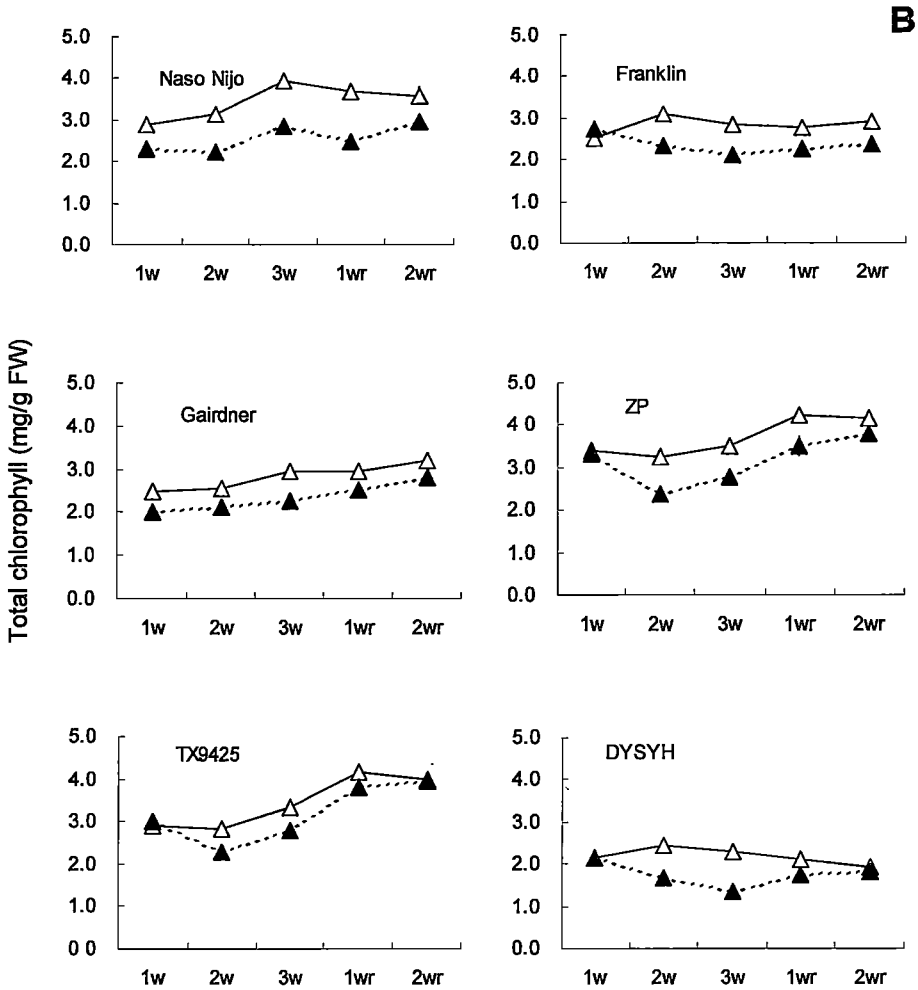

Fig. 4.2B Changes in leaf pigment composition in response to waterlogging (WL) and subsequent drainage in 6 barley genotypes grown in grey Vertosol soil. Data are means ± SE (n=8). Note that in most cases, the error bars are smaller than the symbols.

Chlorophyll content was significantly affected by waterlogging (Fig. 4.2A and B; Table 4.2). As Chl *a* and *b* were affected in a similar way (data not shown), the results presented here are for total chlorophyll. In Experiment 1, chlorophyll content in waterlogged plants was lower than that in the control plants in all genotypes. After 3 weeks waterlogging, there was a significant difference between stressed plants and the control for all cultivars, the reduction in chlorophyll content being most pronounced for Naso Nijo and DYSYH, followed by Franklin and ZP, with

the least difference being for TX9425 and Gairdner (Fig. 4.2A). In Experiment 2, chlorophyll changes followed a very similar trend (Fig. 4.2B).

After 2 weeks of drainage, in Experiment 1, chlorophyll content in stressed plants improved in all genotypes, to the level even larger than in control plants for Gairdner and TX9425, with no significant difference for DYSYH and Franklin, but with still the largest reduction for Naso Nijo. In Experiment 2, the chlorophyll content recovered to the control level in TX9425 and DYSYH, but not in other 4 cultivars, with the biggest reduction being in Naso Nijo and Franklin.

4.4.3 Gas Exchange Parameters

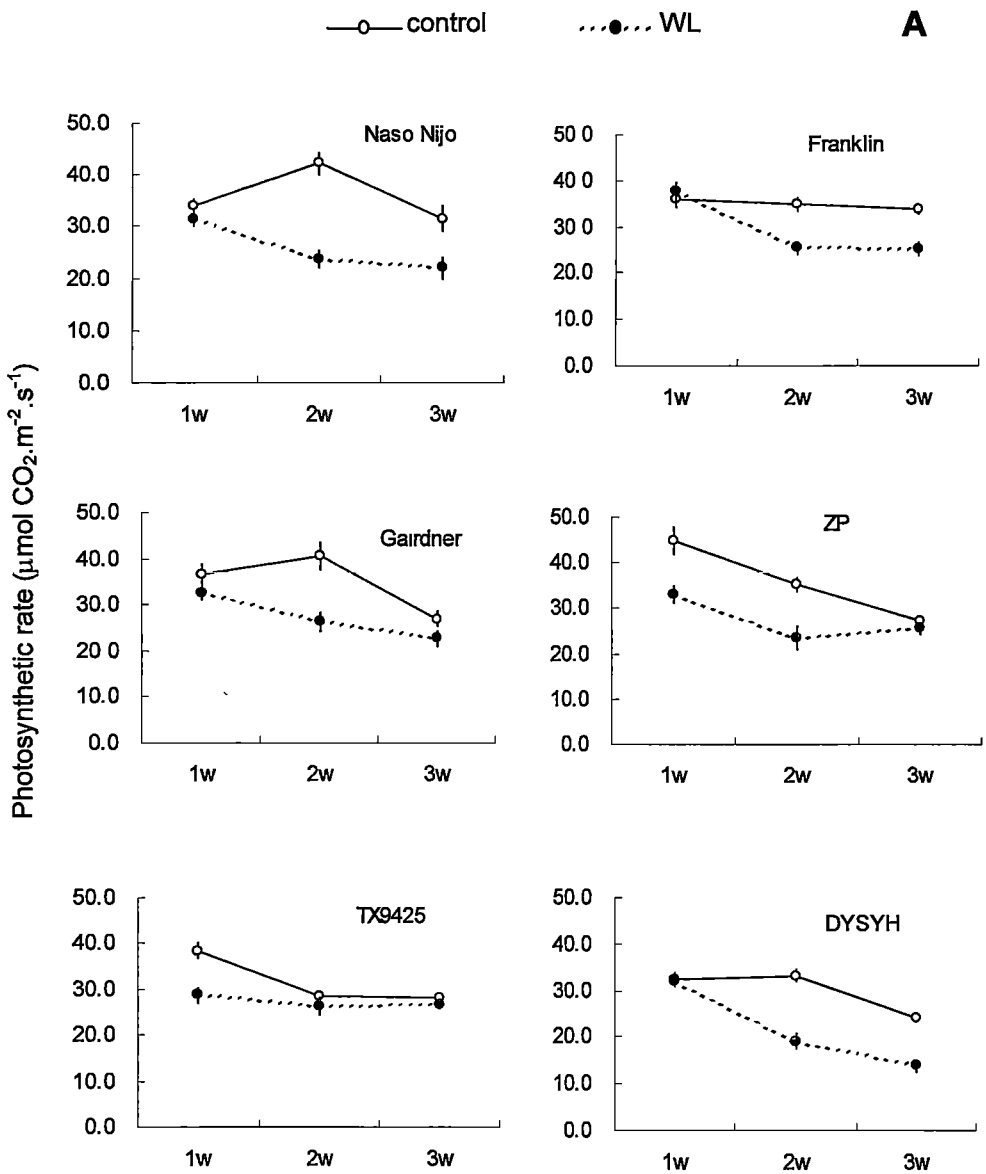

Fig. 4.3A Time-course of response to waterlogging and subsequent drainage in photosynthetic rate (Pn) in waterlogged (WL) and control plants for six genotypes grown in artificial potting mixture. Data are means ± SE (n = 6).

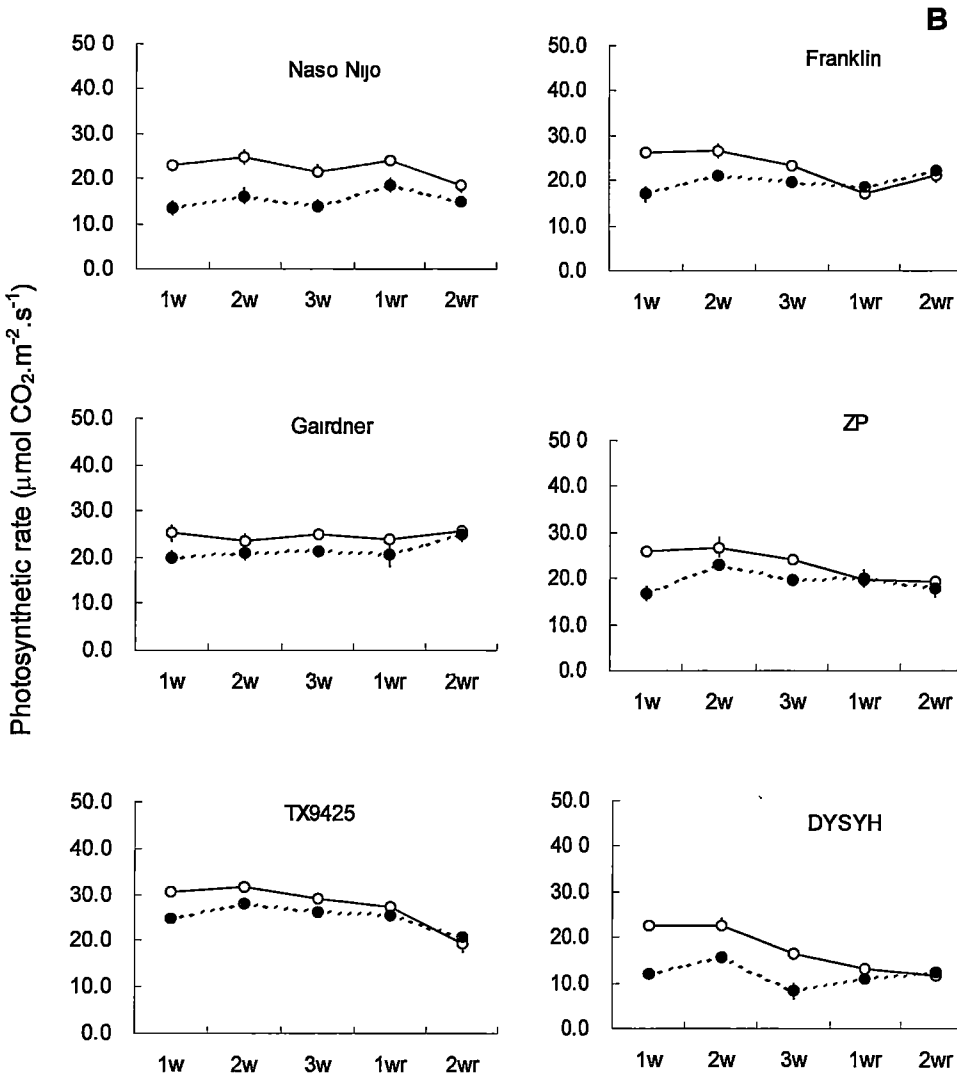

Fig. 4.3B Time-course of response to waterlogging and subsequent drainage in photosynthetic rate (Pn) in waterlogged and control plants for six genotypes grown in grey Vertosol soil. Data are means ± SE (n = 6).

In all cultivars studied, waterlogging caused significant changes in leaf gas exchange characteristics (Fig 4.3A and B). In Experiment 1, photosynthetic rate (Pn) for waterlogged plants was lower than control plants after 1 week of waterlogging in most cultivars (Fig. 4.3A). In the second week of waterlogging, the effects continued to worsen except in TX9425, in which the effects of waterlogging on Pn were alleviated. Interestingly, in the third week of waterlogging, though the Pn in stressed plants still remained lower than the control, the difference began to

decline in all cultivars. Pn also followed a similar trend in Experiment 2, with the largest reduction in all cultivars happening after one week waterlogging and noticeable alleviation of Pn values in the following period of waterlogging (Fig. 4.3B). After 3 weeks waterlogging, under both experimental conditions, the largest adverse effects were shown in DYSYH and Naso Nijo, and the least effects in TX9425.

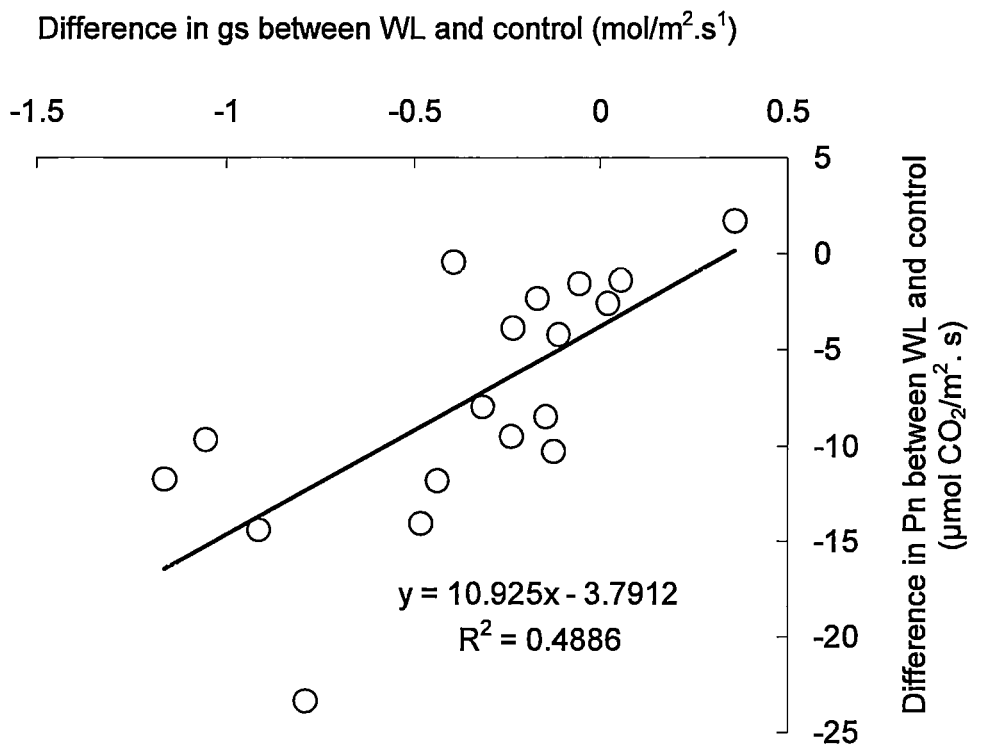

Fig. 4.4. Relationship between changes in photosynthetic rate and stomatal conductance of CO_2 between waterlogged and control plants.

After 2 weeks recovery in Experiment 2 (no Pn recovery data were collected in Experiment 1), Pn recovered in all cultivars, and there was no significant difference between the waterlogged plants and the control except in Naso Nijo.

Stomatal conductance of CO_2 (gs) and transpiration rate followed similar trends after waterlogging (data not shown). In Experiment 1, there was also a rather strong

relationship between gs and Pn, expressed as the differences between waterlogged and control plants in each case (r^2=0.49) (Fig. 4.4).

4.4.4 Chlorophyll fluorescence

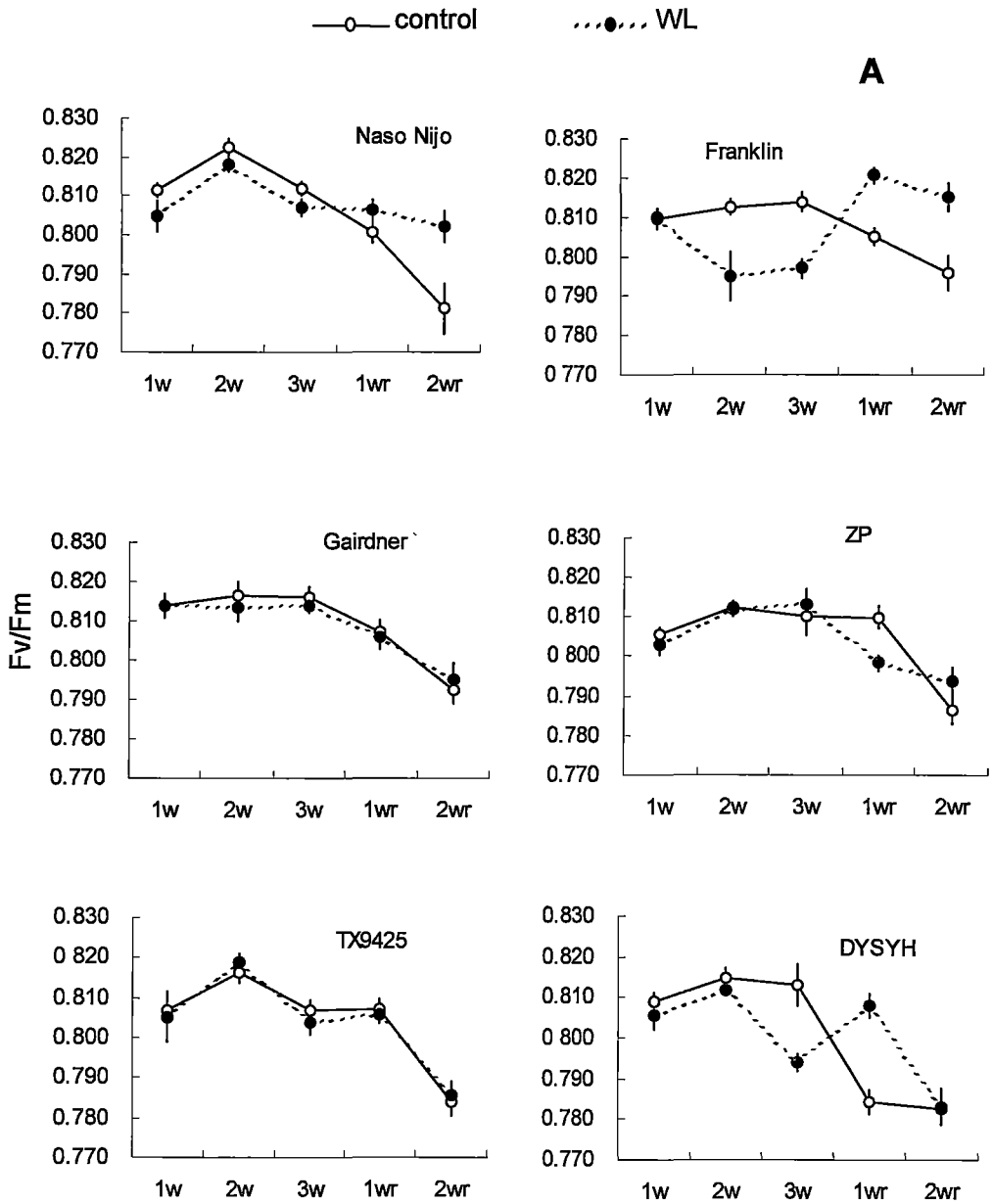

Fig. 4.5A Fv/Fm values (means ± SE; n = 10) for waterlogged and control in barley leaves, after waterlogging and a subsequent drainage, for plants grown in artificial potting mixture.

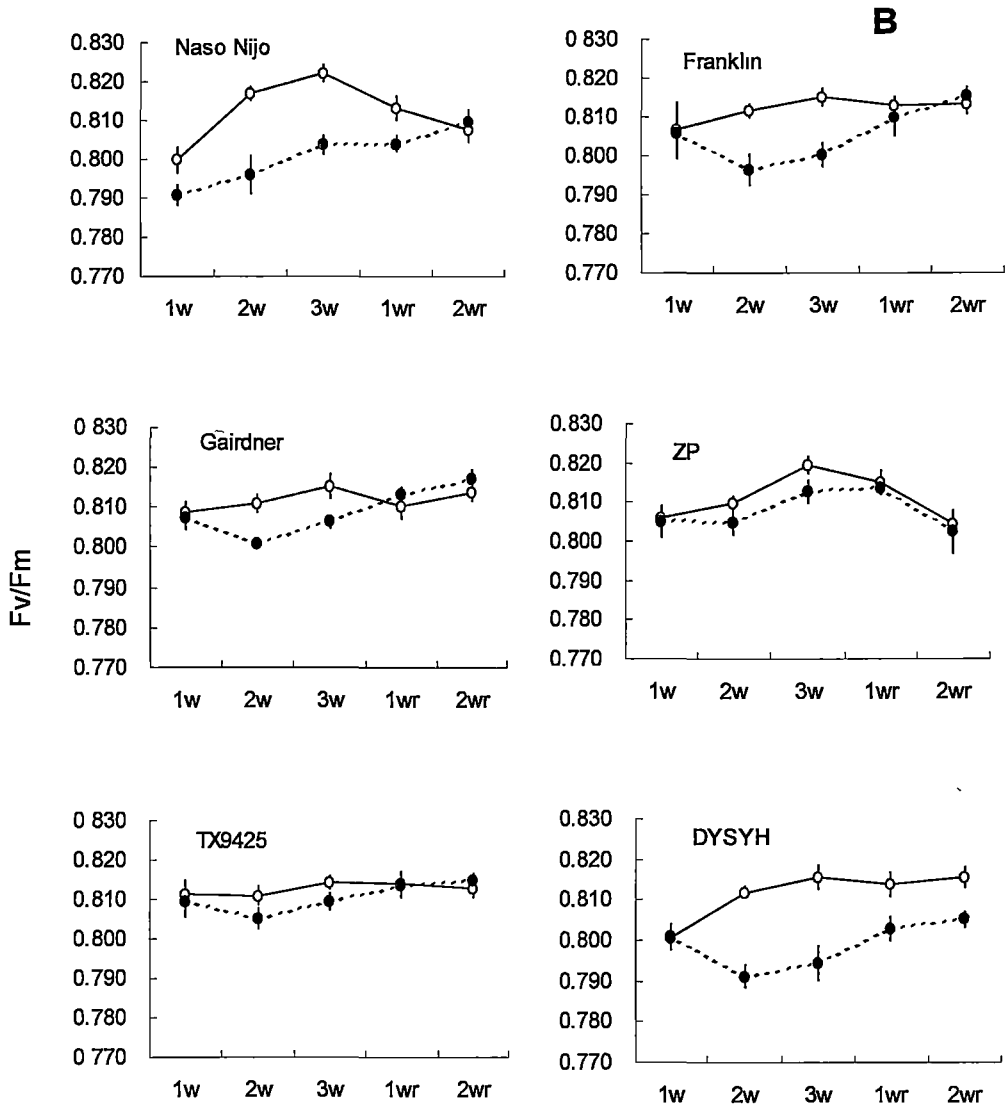

Fig. 4.5B Fv/Fm values (means ± SE; n = 10) for waterlogged and control in barley leaves, after waterlogging and a subsequent drainage, for plants grown in grey Vertosol soil.

Chlorophyll fluorescence characteristics were significantly affected by waterlogging. There was a substantial decrease in Fo and Fm for stressed plants compared with the control by the second week of waterlogging in both experiments (data not shown). After drainage, both Fo and Fm in stressed leaves jumped to values larger than the control.

Different genotypes showed different responses of Fv/Fm to waterlogging (Fig. 4.5A and B). In Experiment 1, significant (P = 0.05; Table 4.2) reduction in Fv/Fm (as compared with control) was observed in some cultivars during waterlogging. The largest Fv/Fm decline was measured in DYSYH and Franklin cultivars two to three weeks after waterlogging. No significant differences existed after 3 weeks of waterlogging for TX9425, ZP and Gairdner (Fig. 4.5A; Table 4.2). Under the more severe conditions of Experiment 2, all cultivars showed more significant reduction in Fv/Fm (P=0.05; Table 4.2) after 2 and 3 weeks waterlogging. The largest reduction was in DYSYH, Naso Nijo, and Franklin, followed by Gairdner, ZP, and the least adverse effects were found for TX9425 (Fig. 4.5B). The difference between waterlogged plants and the control in DYSYH and Franklin was 4-5 times larger than in TX9425.

After 2 weeks of drainage, in Experiment 1, Fv/Fm in stressed plants was higher than in the control in all genotypes, especially in Naso Nijo and Franklin. In Experiment 2, all genotypes also showed complete recovery in Fv/Fm, except in DYSYH which was still much lower in stressed plants compared with the control.

4.4.5 Root morphology and anatomy

In order to provide insight into the mechanisms underlying waterlogging tolerance in barley, the root morphology and anatomy was investigated in TX9425 and Naso Nijo in Experiment 1 (two apparently contrasting cultivars according to the data). Fig. 4.6 shows the typical barley roots after 3 weeks of waterlogging. For both TX9425 and Naso Nijo, the seminal roots partly died and the adventitious roots were produced at the shoot base during and after waterlogging (Fig. 4.6), however, in the control plants, most of the roots are seminal roots.

The length of the longest root was significantly reduced (Fig. 4.7A), while total length of adventitious roots per plant were significantly increased in both TX9425 and Naso Nijo as a result of 3 weeks of waterlogging (Fig. 4.6 and 4.7B). In

TX9425, more adventitious roots were produced in waterlogged plants compared with in Naso Nijo (Fig. 4.6 and 4.7).

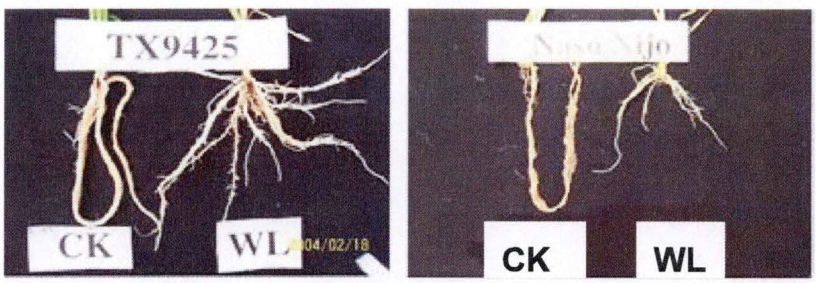

Fig. 4.6. Typical root morphology of TX9425 (left panel) and Naso Nijo (right panel) after 3 weeks of waterlogging. CK-control, WL-waterlogged.

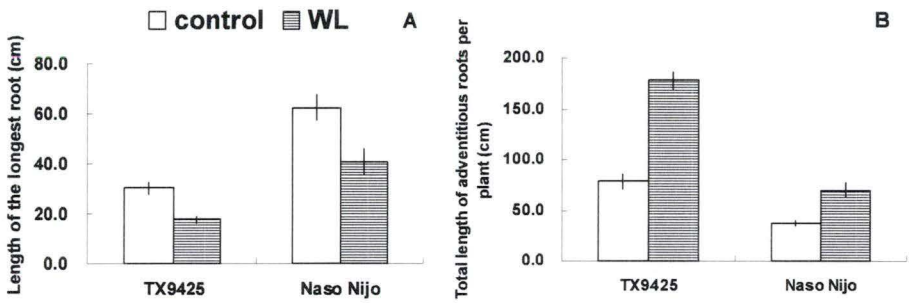

Fig. 4.7. Effects of 3 weeks of waterlogging on the change of root characteristics. (A) the length of the longest root; (B) total length of adventitious roots per plant. Data are means ± SE (n=20)

In adventitious roots, cortical cell breakdown and the formation of air channels (aerenchyma) were observed from 0.5 cm behind the root tip and persisted along the entire adventitious root axis in both genotypes after 3 weeks of waterlogging (Figure 4.8A; Fig. 4.9C and D). No aerenchyma was found in the root of drained plants (Fig. 4.9A and B).

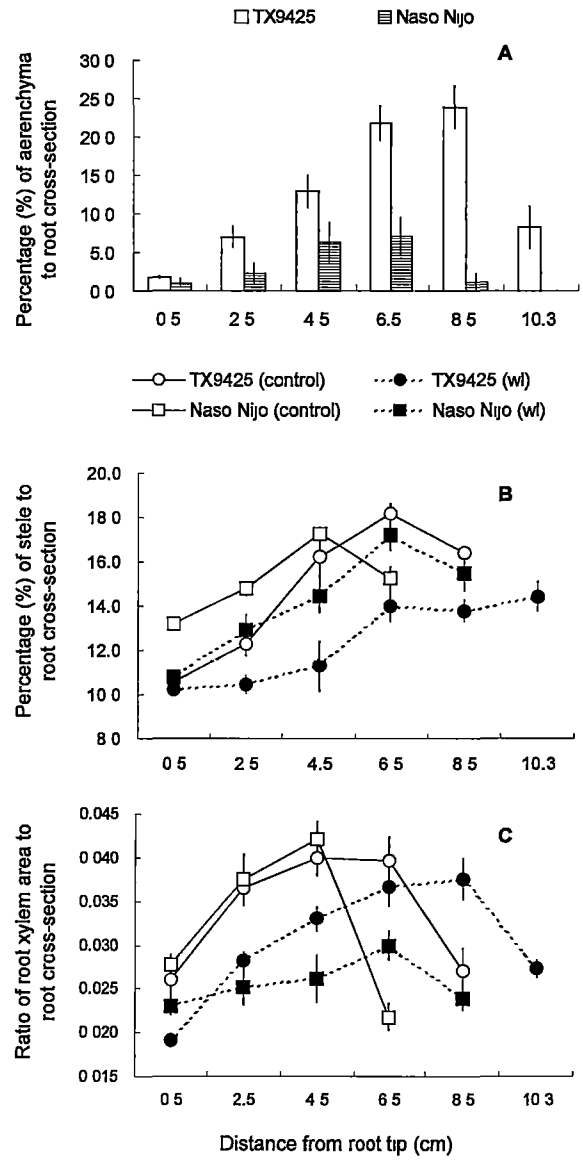

Fig. 4.8. Effects of waterlogging on root anatomy characteristics in Experiment 1. (A) percentage of aerenchyma area in cortex to root cross-section in adventitious roots of waterlogged plants after three weeks of waterlogging; (B) percentage of stele area to root cross-section area in adventitious roots after three weeks of waterlogging; (C) ratio of root xylem area to root cross-section in adventitious roots after three weeks of waterlogging. Average length (cm) of adventitious roots in aerated or hypoxia solutions used for anatomy analysis were: TX9425, 8.5±0.3cm and 10.2±0.4cm; Naso Nijo, 6.5±0.2cm and 8.5±0.4cm, respectively. Data are means ± SE (n = 5).

The percentage of aerenchyma to root cross-section area in TX9425 along the entire adventitious root was clearly larger than that in Naso Nijo (Fig. 4.6A). Aerenchyma at 0.5 cm behind the adventitious root tip accounted for only 1.7% and 1.0% of the root cross-section area for TX9425 and Naso Nijo, respectively. The proportion increased along the root, reaching a maximum of 23.9% and 7.1% at 8.5 cm and 6.5 cm behind the adventitious root tip for TX9425 and Naso Nijo, with an average root length of 10.3 cm and 8.5 cm, respectively. Both maxima occurred at about 2 cm from the root-shoot junction. At the root base, it reduced to 8.2% and 1.1% in TX9425 and Naso Nijo, respectively.

After 3 weeks of waterlogging, the percentage of the root cross-section area occupied by the stele was significantly reduced for both genotypes compared with the value in the control plants, especially in TX9425 (Fig. 4.6B). The ratio of the root xylem area to the root cross-section area also significantly decreased in adventitious roots of waterlogged plants compared with the control. However, it was more adversely affected in Naso Nijo compared with the control (Fig. 4.6C). The ratio between stele and cortex area at 4.5cm from the root tip dropped from 22.2% to 18.8% for TX9425 and from 24.3% to 17.5% for Naso Nijo in control and waterlogged roots, respectively.

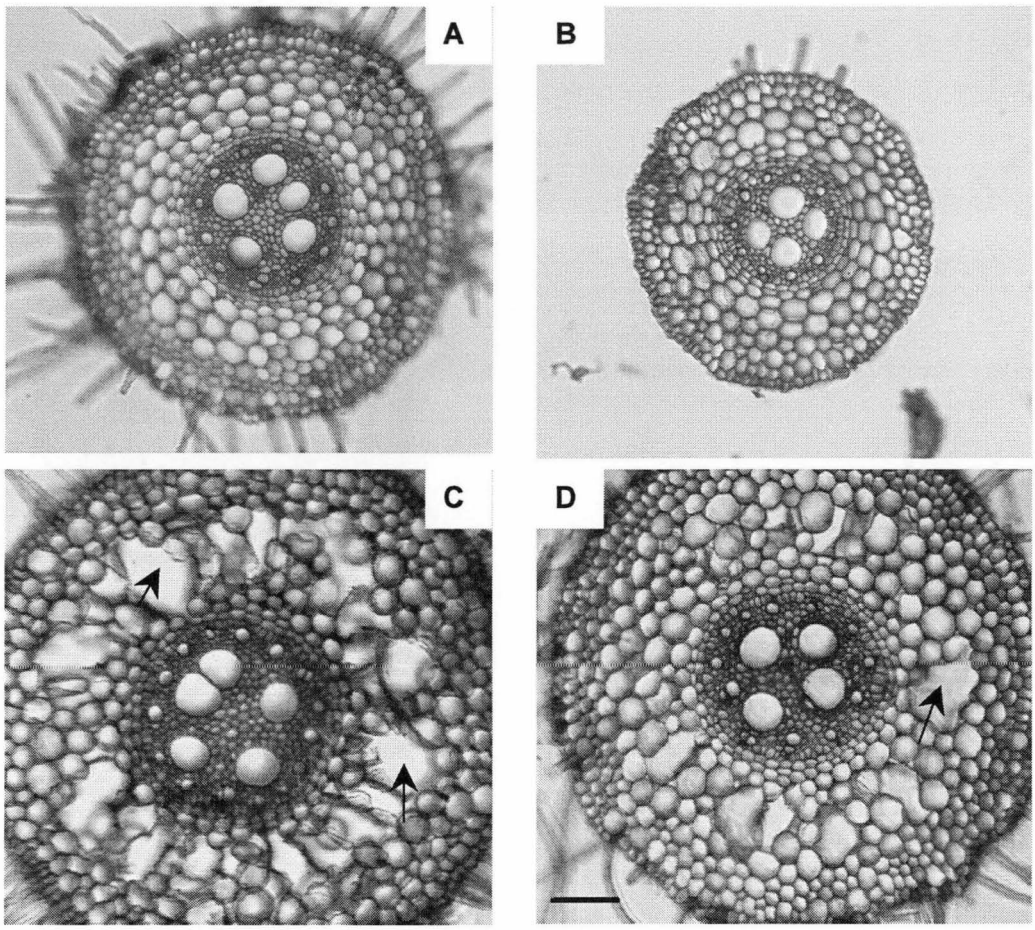

Fig. 4.9. Light micrographs of transverse sections of adventitious roots, showing aerenchyma at 4.5 cm behind the root tip in (C) TX9425 and (D) Naso Nijo roots after three weeks of waterlogging. No aerenchyma was observed in adventitious roots of (A) TX9425 and (B) Naso Nijo in aerated plants. Bar = 100 µm.

4.5. Discussion

Six barley genotypes in this experiment differed not only in their responses to waterlogging but also in the recovery of growth once the soil was drained. In both experiments, after 3 weeks waterlogging, Naso Nijo (originating from Japan) was most severely affected in both relative and absolute root and shoot growth, whereas the relative growth of ZP and TX9425 (both from China) were least affected (Fig. 4.1). Although the limited number of cultivars used in this study does not make it

possible to establish a correlation between the country (region) of origin and a degree of waterlogging tolerance in barley, data from literature suggest that, at least for some other stress responses, such correlation may exist (Kaneko *et al.* 2001). Qureshi *et al.* (1990) suggested that the relative shoot dry weight of wheat seedlings could be used as the best criterion to judge the relative tolerance of a genotype. Drew (1991) found that root systems of waterlogging-intolerant plants undergo large reductions in growth under waterlogged conditions, while tolerant plants initiate vigorous adventitious roots that proliferate abundantly and thus exploit the rooting medium more thoroughly. The present experiments also showed that accompanying the death of seminal roots upon waterlogging, more adventitious roots were produced in waterlogged plants of TX9425 than Naso Nijo (Fig. 4.6 and 4.7).

Some investigators (Krizek 1982, Malik *et al.* 2001) suggested that the ability to recover from transient waterlogging or hypoxia of the root system was crucial to evaluate the waterlogging tolerance of a plant. After the 2-week recovery in Experiment 1, root growth rapidly recovered to larger than or close to that of the control plants in most cultivars except in Naso Nijo. Meanwhile, the shoot growth did not recover to the value of the control for any of the genotypes studied, between 33% and 74%, with TX9425 the highest and Naso Nijo lowest (Fig. 4.1A). This relatively greater root recovery was in accordance with results of Malik *et al.* (2001) who found that carbon was preferentially allocated to root growth during the recovery period. In mungbean, active growth of adventitious roots might also have played a role in recovery from the damage due to waterlogging (Ahmed *et al.* 2002). In Experiment 2, all cultivars showed less recovery compared with plants in the first experiment. Gairdner and TX9425 showed the highest relative root growth (around 50%) and Naso Nijo was the lowest (21%), while TX9425 showed the highest relative shoot growth and Naso Nijo the lowest (Fig. 4.1B).

In general, the adverse effects of waterlogging on shoot and root growth in dark grey Vertosol soil in Experiment 2 were more severe than on plants grown in 'artificial' potting mix in Experiment 1. Also, plants grown in dark gray Vertosol soil showed less recovery. In both experiments, TX9425 and ZP showed better relative growth than other cultivars upon waterlogging and TX9425 also showed better relative growth after recovery. In physiological studies, waterlogging tolerance is defined as either survival or the maintenance of high growth rates under waterlogging relative to non-waterlogged conditions, which contrasts with the agronomic definition of waterlogging tolerance (Setter and Waters 2003). From the agronomic point of view, waterlogging tolerance is the maintenance of relatively high grain yields under waterlogged relative to non- or less- waterlogged conditions. From this point, ZP is the most waterlogging tolerant in this study as it showed the highest absolute biomass after 3 weeks waterlogging stress and the following recovery under both soil conditions. So among these 6 cultivars, ZP is the most suitable for immediate use for agronomists. However, it is generally accepted that high yield of some genotypes on recovery from waterlogged environments may have nothing to do with waterlogging tolerance, while highly tolerant lines may have been discarded simply because they are low yielding genotypes (Setter and Waters 2003). Keeping this in mind, I regard TX9425 as the most valuable to be used in further breeding programs, as having the best relative growth and recovery compared with the control plants.

Upon waterlogging, photosynthetic CO_2 assimilation rate (Pn) was lower than that of the control (Fig. 4.3). This has been shown in different plant species (Batzli and Dawson 1997, Meyer *et al.* 1987, Talbot *et al.* 1987). The decrease in net photosynthesis may be due to reductions in leaf water potential, stomatal conductance, amount or activity of photosynthetic enzymes and chlorophyll (Huang *et al.* 1994a, Huang *et al.* 1994b). Some investigators (Castonguay *et al.* 1993, Limpinuntana and Greenway 1979, Plaut *et al.* 1987) suggested that one possible factor in the reduction of photosynthesis in plants grown in waterlogged soil was

the accumulation of carbohydrates in leaves, which could suggest a feedback inhibition of photosynthesis. Others (Luxmoore and Stolzy 1969) reported that reduced net photosynthesis in plants subjected to hypoxia might be due to reduced respiratory activity in the roots implying a feed back mechanism when the sink for photosynthates was limited. Results in this study showed that differences in photosynthetic rate (Pn) between waterlogged and control plants were correlated with differences in stomatal conductance (g_s) of CO_2 ($r^2 = 0.49$; Fig. 4.4). It appears possible that closure of stomata may have accounted in part for the reduced photosynthetic rate during the waterlogging in this experiment.

Interestingly, in both experiments, by the third week of waterlogging, the reduction of Pn was significantly alleviated in all genotypes (Fig. 4.3). Under both experiments, the largest adverse effects were shown in DYSYH and Naso Nijo, and the least effects in TX9425. The data is in accordance with the result of Smith and Moss (1998) who found that in *Aster pilosus*, significant recovery of stomatal conductance occurred by week 4 during flooding. Such recovery is a clear indication of plant adaptation to hypoxia and might be attributed to several factors including development of stem and root aerenchyma in existing roots which improves the supply of O_2 to the roots (Drew *et al.* 1979a, Gries *et al.* 1990, Moon *et al.* 1993). In addition, production of new roots with greater proportion of aerenchyma tissue (Sena Gomes and Kozlowski 1980, Wenkert *et al.* 1981), and/or improved capacity of existing roots for anaerobic respiration (Daugherty *et al.* 1994) may be also factors contributing to waterlogging tolerance in barley. Indeed, the data suggested that the differential responses of two apparently contrasting cultivars in this study, TX9425 and Naso Nijo, may be due to the changes in root anatomy induced by waterlogging (Fig. 4.8 and 4.9) and, specifically, by a significantly higher fraction of aerenchyma tissue in TX9425 roots. TX9425 also showed a larger reduction in the percentage of the root cross-section area occupied by the stele along the entire adventitious root of waterlogged plants compared with the control than Naso Nijo. McDonald *et al.* (2002) suggested that roots with a

small stele volume relative to the whole root would show enhanced longitudinal O_2 diffusion due to (i) a proportionally lower O_2 consumption within the root, and (ii) a potentially greater cortex volume, the tissue in which aerenchyma is usually formed. The ratio of the root xylem area to the root cross-section area also significantly decreased in adventitious roots of waterlogged plants compared with the control, and it was more adversely affected in Naso Nijo than TX9425. The decrease of the ratio could lead to a relative reduction in root axial conductance for water movement (Huang *et al.* 1994b).

Chlorophyll content (Chl $a + b$) was reduced significantly for all cultivars after 3 weeks waterlogging (Fig. 4.2), with the most pronounced reduction in Naso Nijo, and least pronounced in TX9425 and Gairdner in both trials. When followed by two weeks of recovery, TX9425 showed the best recovery and Naso Nijo the worst in both trials. Ashraf and Chishti (1993) found that the reduction in the percentage chlorophyll content was more pronounced in relatively waterlogging-sensitive lentils compared with waterlogging-tolerant accessions. Talbot *et al.* (1987) also found a greater reduction in the chlorophyll contents of the water-sensitive *Salix caprea* compared with the waterlogging-tolerant species *S. cinerae*. Ashraf and Chishti (1993) suggested that a decrease in the chlorophyll content of all lentil accessions after waterlogging might be related to the reduction in the photosynthetic activity.

Dark-adapted values of Fv/Fm reflected the potential quantum efficiency of PSII and were used as a sensitive indicator of plant photosynthetic performance, with optimal values around 0.83 measured for most plant species (Bjorkman and Demmig 1987, Johnson *et al.* 1993, Shabala 2002). It was found that the maximal quantum efficiency Fv/Fm was reduced after imposing waterlogging stress on oaks (Wagner and Dreyer 1997), wheat (Webb and Fletcher 1996) and *Alnus cordata* (Percival *et al.* 1998). However others have suggested that waterlogging stress may have no effects on the primary photochemistry of PSII studies in *Boltonia decurrens*

(Smith and Moss 1998). In this study, different genotypes showed different responses of Fv/Fm to waterlogging and subsequent drainage (Fig. 4.5). Under both experiments, waterlogged plants in TX9425 showed the least reduction in Fv/Fm compared with the control plants, while DYSYH, Franklin and Naso Nijo showed a much bigger reduction after 3 weeks of waterlogging. After 2 weeks of drainage, Fv/Fm increased for all genotypes, although to different degrees. The adverse effects of waterlogging on Fv/Fm for plants grown in dark grey Vertosol soil was much more obvious than on plants in potting mixture. The difference between the control and waterlogged plants in DYSYH and Naso Nijo was 4-5 times higher than that in TX9425 for plants grown in dark grey Vertosol.

In summary, from a physiological point of view, in searching for characteristics that could be incorporated into breeding programs, TX9425 appears to be the most suitable candidate. In addition to above characteristics, TX9425 also had the highest water use efficiency (WUE) (as judged by the ratio between Pn and transpiration; data not shown). Its small overall biomass, even in controls, means however that it cannot be recommended for immediate use by agronomists, if high yield potential is of primary importance. ZP, Gairdner, DYSYH and Franklin were intermediate, and Naso Nijo was most sensitive to waterlogging. The characteristics that contribute to the better growth under waterlogged conditions and the rapid recovery after drainage for TX9425 included the least pronounced reduction of photosynthetic rate, chlorophyll content and chlorophyll fluorescence and a rapid recovery after drainage for these characteristics. Among these, the total chlorophyll content and changes in Pn values, were the most responsive in both soil types (Figs. 4.2 and 4.3) and may be the best techniques to screen smaller number of lines. Their use for large-scale screening, when thousands of leaf samples need to be analysed is, however, offset by the significant amount of time required for analysis. In this case, chlorophyll fluorescence parameters (specifically, Fv/Fm ratio) may be recommended. Screening should be done in soil rather than potting mix, as the adverse effects in Vertosol soil were so much more obvious, and closer to the real

situation on farms. With only a few seconds per sample required, the chlorophyll fluorescence technique appears to be the most suitable for large-scale programs when selecting barley genotypes for waterlogging tolerance. To further understand the underlying physiological mechanisms, more specific studies at cellular and tissue levels are needed.

Chapter 5. Microelectrode ion and O_2 flux measurements reveal differential sensitivity of barley root tissues to hypoxia*

5.1. Abstract

Hypoxia-induced changes in net H^+, K^+ and O_2 fluxes across the plasma membrane of epidermal root cells were measured using the non-invasive microelectrode MIFE system in elongation, meristem, and mature root zones of two barley (*Hordeum vulgare* L.) cultivars contrasting in their waterlogging tolerance. The ultimate goal of this study was to shed light on the mechanisms underlying effects of waterlogging on plant nutrient acquisition and mechanisms of waterlogging tolerance in barley. Measurements revealed that functionally different barley root zones have rather different O_2 requirements, with the highest O_2 influx being in the elongation zone of the root at about 1 mm from the tip. Oxygen deprivation has qualitatively different effects on the activity of plasma membrane ion transporters in mature and elongation zones. In the mature zone, hypoxic treatment caused a very sharp decline in K^+ uptake in the waterlogging sensitive cultivar Naso Nijo, but did not reduce K^+ influx in the waterlogging tolerant TX9425 cultivar. In the elongation zone, onset of hypoxia enhanced K^+ uptake from roots of both cultivars.

Pharmacological experiments suggested that hypoxia-induced K^+ flux responses are likely to be mediated by both KIR and NSCC channels in the elongation zone, while in the mature zone KOR channels are the key contributors. Overall, my results suggest that oxygen deprivation has an immediate and substantial effect on root ion flux patterns, and that this effect is different in waterlogging sensitive and tolerant cultivars. It remains to be answered in future studies to what extent this difference in ion flux response to hypoxia is a factor conferring waterlogging tolerance in barley.

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5.2. Introduction

Nutrient acquisition by roots critically depends on availability of O_2. Under normal soil conditions, water dissolves about 230 $mmol \cdot m^{-3}$ O_2. Hypoxia occurs when the O_2 level in the soil solution falls below 50 $mmol \cdot m^{-3}$ (Grichko and Glick 2001). As O_2 serves as the terminal electron acceptor of mitochondrial electron transport, lack of O_2 effectively blocks aerobic respiration and ATP synthesis in this organelle (Greenway and Gibbs 2003, Pradet and Bomsel 1978). Shortly after the onset of flooding, soil micro-organisms consume all of the available O_2, and toxic compounds begin to accumulate in the soil (Grichko and Glick 2001).

Oxygen stress (either anoxia or hypoxia) in roots is accompanied by a rapid decline in adenylate energy status of plant tissues, depletion of carbohydrate reserves, disturbance to pH homeostasis, and significant ultrastructural changes (Roberts *et al.* 1984a, Saglio *et al.* 1980). As a result, significant hormonal changes are induced, cell integrity is disrupted, and nutrient acquisition is impaired (Atwell and Steer 1990, Drew 1988). It is not clear, however, to what extent these processes differ between different functional zones of the root. It was shown that net losses of K^+ and Cl^- were faster in expanding than in expanded root tissues after several days of oxygen deprivation (Greenway *et al.* 1992). Is this difference attributable to different O_2 requirements between growing and expanded root tissues as shown by recent experiments on *Vitis* roots (Mancuso and Boselli 2002, Mancuso *et al.* 2000)? It is known that both hypoxia and anoxia rapidly depolarise the plasma membrane potential of higher plants by about 50mV (Buwalda *et al.* 1988), presumably as a result of inhibition of electrogenic H^+ pumps (Greenway and Gibbs 2003). This membrane depolarization may control ion transport through voltage-gated ion channels in root plasma membranes. It remains to be answered, however, whether all ion transporters are affected to the same extent by hypoxia, or whether some of them are more sensitive to O_2 deprivation. Also still unexplored is the spatial profile of the observed ion "leak" along the root axis.

Not only membrane voltage but also other intracellular factors may modify ion channel permeability. Among the most important are cytosolic pH (Blatt and Armstrong 1993), intracellular ATP (Shimmen and Tazawa 1977) and cytosolic free Ca^{2+} concentration (Schroeder and Hagiwara 1989). Changes in these factors under hypoxia have been reported. Anaerobiosis caused significant cytosolic acidification of the maize root tip within 20 min after transfer to an anaerobic environment (Roberts *et al.* 1984b). In maize root tip cells, cytosolic pH decreased sharply, from pH 7.5 to 6.9, in response to oxygen deprivation within the first 10 min, but then quickly stabilized at pH 7.1 over the next 10-15 min (Fox *et al.* 1995, Saintges *et al.* 1991). It is also believed that gene expression and physiological changes in response to O_2 deprivation are preceded and signalled by an elevation of cytosolic free Ca^{2+}, presumably due to Ca^{2+} release from intracellular stores (Subbaiah *et al.* 1994b). Therefore, it is likely that not only changes in the plasma membrane potential, but also other factors may contribute significantly to gating permeability of various ion channels under hypoxia. Specific details of this process remain obscure.

The aim of this study was to quantify hypoxia-induced changes in net ion fluxes across the plasma membrane (PM) of epidermal root cells in physiologically different root zones (meristem, elongation, and mature regions) in order to shed light on the ionic mechanisms underlying effects of waterlogging on plant nutrient acquisition. Non-invasive microelectrode ion flux measurements (MIFE) were complemented by measurements of net O_2 fluxes to the root surface. Two barley cultivars, contrasting in their waterlogging tolerance (Chapter 4 and Pang *et al.* 2004), were used. Measurements revealed different O_2 requirements for functionally different plant zones and qualitatively different effects of O_2 deprivation on activity of plasma membrane ion transporters in mature and elongation zones of barley roots. Pharmacological experiments suggest that several types of plasma membrane transporters may mediate root responses to hypoxia. Overall, results suggest that oxygen deprivation has an immediate and substantial

effect on root ion flux patterns, and this effect is different in waterlogging sensitive and tolerant cultivars.

5.3. Materials and Methods

5.3.1 Plant material

Two contrasting barley (*Hordeum vulgare* L.) cultivars, waterlogging tolerant TX9425 and waterlogging sensitive Naso Nijo cultivars (Chapter 4 and Pang *et al.* 2004), were grown hydroponically for 3 to 4 days on a floating mesh in plastic containers above 0.5 L of aerated nutrient solution containing 0.1mM $CaCl_2$ and 0.2 mM KCl under laboratory conditions (temperature + 24 °C; 16 h photoperiod; fluorescent lighting about 150 μmol m^{-2} s^{-1}). Seedlings were used for measurement when their root length was 60 to 80 mm.

5.3.2 Ion flux measurements

Net fluxes of H^+ and K^+ were measured using the non-invasive MIFE system (University of Tasmania, Hobart, Australia). Details on fabrication and calibration of H^+ and K^+ ion selective microelectrodes were described in Section 3.5. Briefly, pulled and silanised microelectrodes with tip diameters of about 3 μm were back filled with appropriate solution (0.15 mM NaCl + 0.4 mM KH_2PO_4 adjusted to pH 6.0 using NaOH for proton electrode; 0.5 M KCl for potassium). The electrode tips were then filled with ionophore cocktails (Fluka; catalogue no. 95297 for H^+; 60031 for K^+). Electrodes were mounted on a three-dimensional electrode holder (MMT-5, Narishige, Tokyo, Japan), positioned with their tips spaced 2-3 μm apart in line. They were calibrated in an appropriate set of standards before and after use (pH from 4.4 to 7.8; K^+ from 0.2 to 10 mM).

In all experiments, basic solution (0.1mM $CaCl_2$, 0.2 mM KCl, pH 5.5 unbuffered) was used. One hour before measurements, 5 mL basic solution was added to a plexiglass measuring chamber (100 mm long, 30 mm deep, and 4 mm wide). A

seedling was taken from the growth container and placed immediately into the measuring chamber. The root was immobilised in the horizontal position by fine plexiglass partitions 5 mm above the floor of the chamber essentially as described by Shabala & Knowles (2002). The chamber was put onto the microscope stage in the Faraday cage and the plant was allowed to adapt to experimental conditions. During measurements the chamber was moved vertically in a square-wave manner with a 10-s cycle so that the electrode tips moved between two points 50 and 100 μm above the tissue. After measuring steady state fluxes for five minutes, 5 ml of 0.1% (w/v) agar solution pre-bubbled with high purity N_2 (BOC Gases, 032G) was added into the chamber with O_2 concentration around 20 μmol L^{-1} to simulate waterlogging (Wiengweera *et al.* 1997), resulting in a final concentration of 0.05% agar in the bath solution. About 2 min were required for the above procedure to be completed and unstirred layer conditions to be regained. This period of time was discarded from the analysis and appears as a gap in the figures.

In some experiments, measurements in the root apex were performed on decapped roots. Decapping is a standard procedure to avoid confounding effects of gravitropism (Bjorkman and Cleland 1991). The typical gravitropic curvature is first detectable within 30 min (Bjorkman and Leopold 1987) after gravistimulation, and gravitropic response is usually complete within 2-3 h (Bjorkman and Leopold 1987, Evans 1991). This is within the timescale of my measurements and might potentially affect steady-state ion fluxes. For this purpose, the root cap was peeled gently from opposite sides of the root tip and cut off with a scalpel blade essentially as described before (Shabala and Newman 1997b, Shabala *et al.* 1997). Decapped roots placed horizontally showed all the typical features of circumnutational movement and exhibited the same rate of growth (20 to 35 μm/min) as intact roots indicating that no damage to root was done by the root cap removal.

Finally, in some experiments excised roots were used for measurements in order to eliminate the possibility of internal oxygen transport. These were of the same length

as roots of intact plants and were measured in precisely the same conditions as described above.

5.3.3 O_2 flux measurements*

Oxygen flux measurements were performed using a commercially available O_2 microsensor (Unisense, OX10 type, Aarhus, Denmark), which is a miniaturized Clark-type O_2 sensor (10 μm tip diameter) with an internal reference and a guard cathode. Driven by the external partial pressure, O_2 from its environment penetrates the sensor tip membrane and is reduced at the gold cathode surface. The sensor was connected to a high-sensitivity picoammeter (Unisense PA2000, Aarhus, Denmark) whose output voltage was connected to the MIFE system to obtain a graph of voltage *vs* time.

Chemicals in solution move under the influence of chemical forces of diffusion directed towards lower concentration regions. Neutral molecules, with valence $z = 0$, do not have electric forces that are experienced by ions. The basic electrochemical theory (Newman 2001) requires modification for neutral molecules, whose electrochemical potential μ (J mol^{-1}) is:

$$\mu = \mu_0 + RT \ln(\gamma c). \qquad \text{[Equ 1]}$$

The molecular flux J (mol m^{-2} s^{-1}) into the tissue (following Newman 2001, Equ 2) is:

$$J = c\, u\, (\mathrm{d}\mu/\mathrm{d}x). \qquad \text{[Equ 2]}$$

The concentration is c (mol m^{-3}), γ is the activity coefficient, u is the mobility (m s^{-1} per newton mol^{-1}) and x is distance (m) from the tissue.

*The first O_2 flux measurement using the MIFE system was published in *Plant, Cell and Environment* by Zivanovic et al. (2005) 28: 340-352

Mathematically, μ is a function of c, which varies with x,

$$(d\mu/dx) = (d\mu/dc)(dc/dx),$$

and, differentiating Equ 1,

$$(d\mu/dc) = RT/c.$$

Hence the flux can be written

$$J = u\,R\,T\,(dc/dx). \qquad \text{[Equ 3]}$$

This equation is known as *Fick's Law of diffusion*, $J = D\,(dc/dx)$, where the diffusion coefficient $D = u\,R\,T$. Thus for uncharged molecules, unlike the situation for ions, the treatment based on electrochemical potential is identical to diffusion theory based on *Fick's Law*. More details are available at http://www.mife.com.

To stabilise the operation of the sensor before calibration and use, the electrode was pre-polarized by immersing its tip in continuously aerated water, to consume the O_2 in the electrolyte by the sensing cathode and the guard cathode. Electrodes used to measure the concentration of neutral molecules required calibration to obtain a graph of output voltage as a function of concentration. Calibration was performed in well-aerated water for atmospheric O_2 reading and in water vigorously bubbled with high-purity N_2 for zero O_2 reading. This Clark-type O_2 microelectrode responds linearly to changes in O_2 partial pressure. The equation is:

$$V = V_0 + a\,c. \qquad \text{[Equ 4]}$$

The intercept is V_0 and the slope of the graph is a.

Thus, $(dV/dc) = a$, or $dc = dV/a$, so the flux Equ 3 becomes:

$$J = u\,R\,T\,(dV/dx)/a. \qquad \text{[Equ 5]}$$

For a cylindrical root with radius r, dx in Equ 5 is replaced by:

$$dx = r \ln[(r + x + dx)/(r + x)] \qquad \text{[Equ 6]}$$

This equation was used by the MIFE software to calculate net flux of O_2.

5.3.4 O_2 flux measurement protocol

The seedlings used for O_2 flux measurement were grown under the same conditions as for measuring net ion fluxes (see above). One hour before measurement, the plants were transferred to the measuring chamber. One seminal root was selected and fixed horizontally 5 mm above the chamber floor on two fine partitions using a cotton thread to prevent floating. When transient O_2 fluxes were measured, the chamber was half-filled with fresh bathing solution and put onto the microscope stage in the Faraday cage. The O_2 micro-sensor tips moved in a square-wave manner (10 sec half-cycle) between 50 and 100 μm radially from the tissue using a computer-driven hydraulics micromanipulator essentially as described for ion flux measurements. No solution disturbance was caused by electrode movement, as evident by the absence of O_2 fluxes in the absence of plant tissue (data not shown). O_2 flux was measured near the root surface in steady state conditions for 5 min. Then a hypoxia treatment was given. This was achieved by adding N_2 - bubbled agar solution (final concentration 0.05% w/v) to the measuring chamber with O_2 concentration around 20 μmol L^{-1}. To further reduce O_2 reabsorption from the air into the agar solution, a significant part of the measuring chamber surface was covered by a glass lid as suggested by Armstrong *et al.* (2000). When O_2 flux profiles along the root axis were measured under hypoxia conditions, roots were pre-incubated in 0.05% (w/v) agar solution pre-bubbled with N_2 gas for 0.5 to 5h.

5.3.5 Pharmacology

Pre-treatment with inhibitors was done when the root was transferred to the measuring chamber. Orthovanadate (an inhibitor of P-type ATPase), TEACl (a putative K^+ channel blocker) and $GdCl_3$ (used to block the NSCC, non-selective cation channels) were used to modify the activity of selected plasma membrane

transporters. These inhibitors were mixed with the basic solution (0.1mM CaCl2, 0.2 mM KCl) to achieve their final concentrations of 500μM, 20 mM, and 30 μM, respectively.

5.3.6 Statistics

Significance of difference between means was evaluated by the Student's *t*-test.

5.4. RESULTS

5.4.1 Oxygen flux measurements

5.4.1.1. Methodological aspects of hypoxia treatment

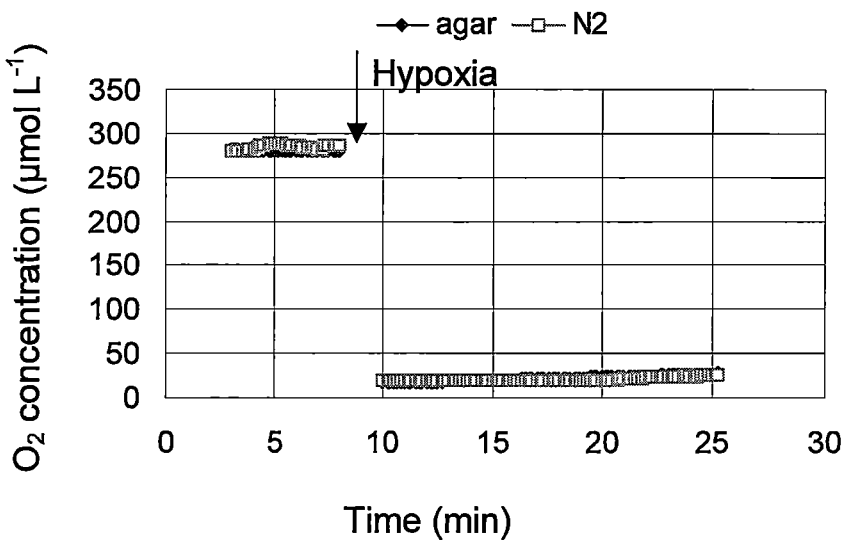

Fig. 5.1. Changes in O_2 levels in basic solution for two different types of hypoxic treatment. In one method (N_2; open symbols), the solution was bubbled with high purity nitrogen for 5 min then covered with a thin layer of silicone oil on the surface. In the other method, agar solution (0.1% w/v) was pre-bubbled with N_2 and mixed with original solution at 1:1 (vol/vol) ratio in the measuring chamber resulting in the final concentration of 0.05% agar in the measuring solution. One typical example (out of 5) is shown.

A convenient method of creating hypoxia in laboratory hydroponics experiments, used by many authors, is flushing the growth solution with N_2 to create the hypoxic environment (Visser *et al.* 1996c, Xia and Roberts 1996). This method, however, cannot be employed when net fluxes are to be measured in experiments, as unstirred layer conditions are required (Newman 2001, Shabala *et al.* 1997). An alternative method is to incubate the root in a dilute agar solution, pre-bubbled with pure nitrogen gas (Wiengweera *et al.* 1997). The presence of the agar prevents

convective streaming within the supporting medium and is expected to maintain a low level of O_2 in the solution near the root. These alternative methods were compared in direct experiments as shown in Fig. 5.1. The O_2 concentration was around 280 μmol L^{-1} in the control (normoxia) solution. Both hypoxia treatments led to identical and very rapid depletion of the O_2 levels in solution down to about 20 μmol L^{-1}. No significant changes in O_2 concentration were observed for at least 20 min in either method (Fig. 5.1). Thus, the use of 0.05% (w/v) agar solution (final concentration) is a reliable and controlled way of imposing hypoxia onto plant roots. This method was used in all my experiments.

5.4.1.2. Vibrating O_2 probe measurements reveal spatial and temporal variations of O_2 fluxes into barley roots

The MIFE technique of non-invasive ion flux measurements (Newman 2001) was further developed in this study (see section 5.3.3) to use a slowly vibrating micro-O_2 probe to measure net O_2 fluxes (Fig. 5.2). Significant ($P = 0.05$) spatial variability in net O_2 uptake was found along the root surface under normoxia conditions (Fig. 5.2A). The highest O_2 influx was measured in the elongation zone of the root at about 1 mm from tip. On average, O_2 fluxes in the root apex were double those in the mature root zone under control conditions (Fig. 5.2A). Hypoxia stress significantly (10-fold; $P = 0.001$) reduced net O_2 uptake in all root zones (Fig. 5.2B). The effect had pronounced time dependence (Fig. 5.2B), with O_2 flux becoming negative (O_2 evolution) in some parts of the mature root zone after 5 h hypoxia. The peak in flux in the elongation zone (at 1 mm distance), observed in normoxia (Fig. 5.2A) was not seen in the hypoxic conditions (Fig. 5.2B).

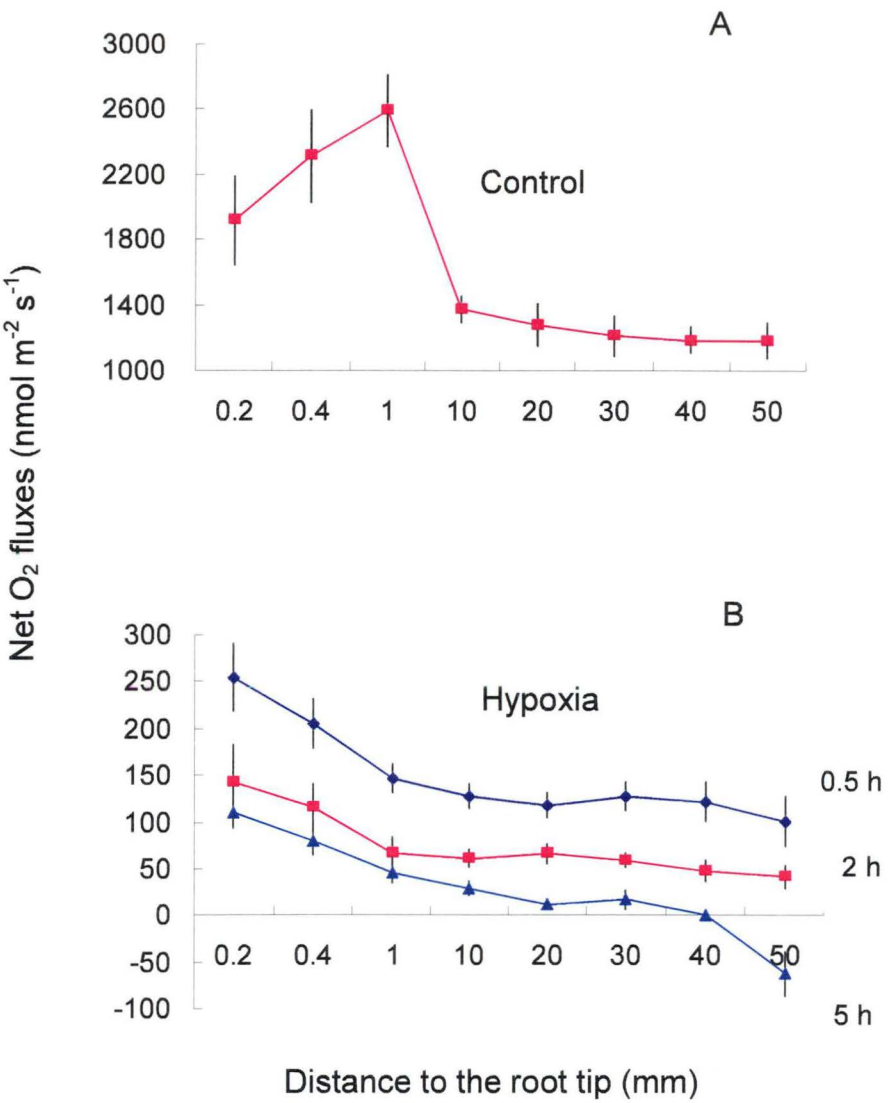

Fig. 5.2. Net O_2 flux profiles along the root axis of Naso Nijo under normoxia (A) and hypoxia (B) conditions. The O_2 micro-sensor tip moved between 50 µm and 100 µm radially from root tissue. Labels of curves in panel B indicate time since onset of treatment. Error bars are SE (n =12).

5.4.1.3. Genetic variation in O_2 flux responses to hypoxia in barley

Table 5.1. Some basic anatomical characteristics and growth rate data of cultivars used in experiments. Statistics are means ± SE (n).

Parameter	*Naso Nijo*	*TX9425*
Average root length at the time of measurement, mm	70 ± 1.9 (12)	68 ± 2.1 (12)
Average root diameter, μm	477 ± 5 (20)	472 ± 8 (20)
Average rate of growth under normoxia conditions, μm/min	33.1 ± 1.6 (14)	25.7 ± 1.3* (15)
Average rate of growth under hypoxia conditions, μm/min	13.5 ± 0.35 (14)	14.3 ± 0.23 (15)
Relative elongation rate under hypoxia, % to control	40.8 ± 2.9 (14)	55.8 ± 2.5* (15)

Note: significant at *P = 0.05 according to Student's *t*-test.

Hypoxia-induced changes in O_2 fluxes and concentration profiles were compared between waterlogging tolerant TX9425 and waterlogging sensitive Naso Nijo barley cultivars. Earlier it was showed that these two genotypes exhibited contrasting growth and photosynthetic responses to prolonged waterlogging stress (Chapter 4 and Pang *et al.* 2004). Measurements of root elongation rates (Table 5.1) also suggested that TX9425 plants were also capable of maintaining significantly (P = 0.05) higher relative elongation rate in short-term (20 min treatment) hypoxic experiments than Naso Nijo (55.8 ± 2.5 % and 40.8 ± 2.9% of the control, respectively; Table 5.1). Other anatomical characteristics such as root length and diameter were not significantly different, except that the rate of root growth of Naso Nijo was higher than that of TX9425 under normoxia conditions (Table 5.1).

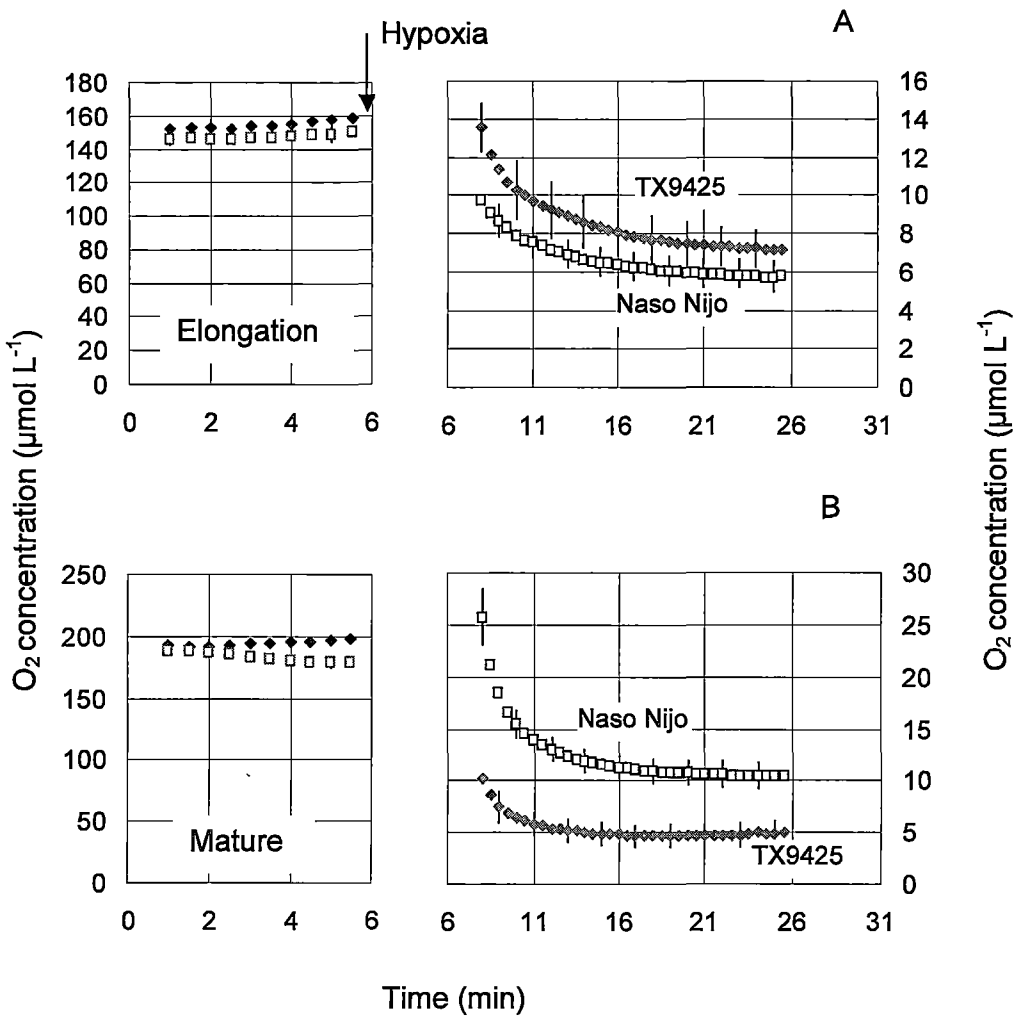

Fig. 5.3. O_2 concentrations measured near the root surface of waterlogging (WL)-tolerant (TX9425, closed symbols) and WL-sensitive (Naso Nijo, open symbols) barley plants in elongation (A) and mature (B) root zones. Please note different scales for O_2 concentrations before and after stress. Error bars are SE (n =10).

Oxygen flux measurements were taken in two functionally different zones: elongation (1 mm from tip) and mature (10 mm from tip). In both cultivars, initial O_2 concentration around the elongation zone was lower than for the mature zone (Fig. 5.3), consistent with my previous observation of higher net O_2 influx in the elongation zone (Fig. 5.2). Hypoxia significantly reduced O_2 concentrations near plant roots. No significant difference between sensitive (Naso Nijo) and tolerant

(TX9425) plants was found in the elongation zone (Fig. 5.3A), while in the mature zone TX roots showed greater reduction in O_2 concentration near the root surface, to about half that of Naso Nijo.

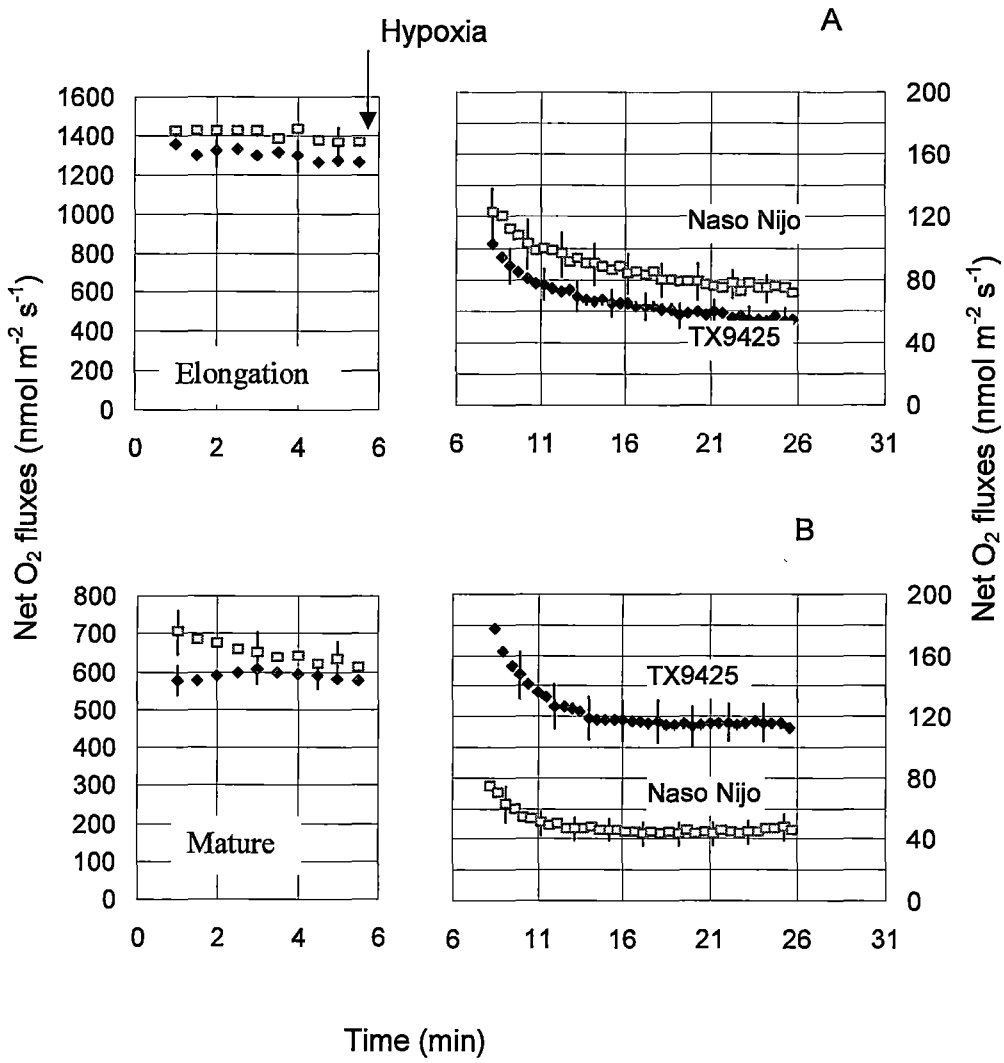

Fig. 5.4. Net O_2 fluxes measured near the root surface of TX9425 (closed symbols) and Naso Nijo (open symbols) in elongation (A) and mature (B) root zones. Please note different scales for O_2 fluxes before and after stress. Error bars are SE (n =10).

Net O_2 flux measurements (Fig. 5.4) were consistent with the above observations. No significant difference was found between the two contrasting cultivars in control

conditions. Net O_2 uptake rate in the elongation zone (1300-1400 nmol m^{-2} s^{-1}) was double that of the mature zone (600-700 nmol m^{-2} s^{-1}) (Fig. 5.4). After hypoxia, the O_2 influx was significantly reduced along the whole root in both cultivars (Fig. 5.4). In the elongation zone, the adverse effect on O_2 uptake was not significantly (P = 0.05) different between cultivars (Fig. 5.4A), while in the mature zone, waterlogging tolerant TX9425 maintained a much higher (3-fold) O_2 uptake than Naso Nijo (Fig. 5.4B).

5.4.2 Ion flux measurements

5.4.2.1. Methodological aspects of ion flux measurements under hypoxic conditions

To my knowledge, there are only a few reports dealing with the use of the ion-selective microelectrode technique to measure fluxes of ions from hypoxia-stressed roots (Rubinigg *et al.* 2002). As a result, many important methodological issues related to application of this technique, have not been adequately addressed. In particular, it remained unknown whether the low concentration agar solution, widely used to simulate hypoxia conditions (Rubinigg *et al.* 2002, Wiengweera *et al.* 1997), will have any confounding effects on microelectrode properties and change the LIX (liquid ion exchanger) selectivity. If it does, what is the threshold agar concentration affecting the behaviour of microelectrodes, and how may these effects be taken into account?

To answer the above questions, ion-selective microelectrodes were calibrated in a set of standards, containing various concentrations of agar. Fig. 5.5A shows one typical example (out of 5) of calibration for a K^+ microelectrode. Three K^+ standards (200, 500 and 1000 µM) and four levels of agar concentration (0, 0.05%, 0.1% and 0.2% w/v) were used. Results showed that the presence of 0.05% agar in the standards did not significantly (P = 0.05) affect electrode characteristics. Higher

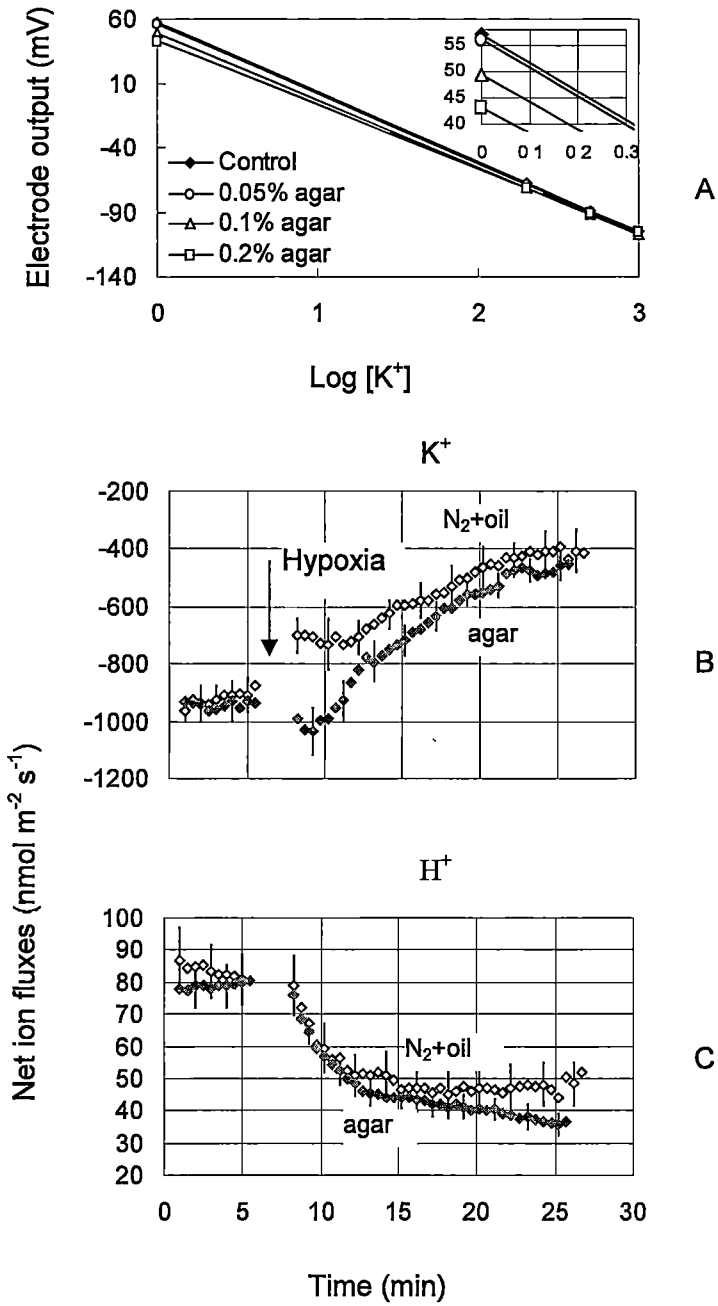

Fig. 5.5. A - a typical example of calibration of K^+ microelectrode in media containing various concentrations of agar (0, 0.05%, 0.1% and 0.2% w/v). B, C – comparison of two different methods of hypoxic treatment on kinetics of net K^+ (B) and H^+ (C) fluxes measured from the elongation zone of Naso Nijo roots. Error bars are SE (n =8).

agar concentrations caused some significant changes in both the slope and the intercept of the K^+ electrode (Fig. 5.5A). Good linear relationship (R>0.999) was observed in all cases.

To my knowledge, no information has been available on whether, or to what extent, the mobility of ions is changed in dilute agar solution. Accordingly, I have compared kinetics of hypoxia-induced ion fluxes from plants measured in 0.05% agar solution with those from plants measured in N_2 pre-bubbled solution (with a thin layer of silicone oil added on the surface to prevent any possible O_2 reabsorption; please refer to Fig. 5.1 for O_2 concentration level). As shown in Fig. 5.5B and C, very similar kinetics of net K^+ and H^+ fluxes was measured from N_2/oil- and agar-treated roots. This confirms that ion mobility in agar solutions is either unaffected by agar in the range of concentrations used or adequately accounted for during calibration.

Overall, the results in this section fully validate the use of the MIFE technique for flux measurements under 0.05% agar-induced hypoxia.

5.4.2.2. Ion flux responses to hypoxia are root-zone-specific

5.4.2.2.1. K^+ fluxes from intact roots

Significant net K^+ uptake was measured into the mature zone in both TX9425 and Naso Nijo cultivars (Fig. 5.6A). Hypoxic treatment caused a very sharp decline in K^+ uptake in the waterlogging sensitive cultivar Naso Nijo, but did not reduce K^+ influx in the waterlogging tolerant TX9425. As a result, a significant difference (P = 0.001) in K^+ flux 10 mins after onset of hypoxia existed between two cultivars.

In contrast to the mature zone, both the elongation (1 mm from the root tip) and meristem (0.3mm) zones showed net K^+ efflux for both cultivars under normoxic conditions (Fig. 5.6B, C). Hypoxia caused gradual decline of K^+ efflux (a shift towards net K^+ influx) in both elongation and meristem zones in both cultivars. Twenty minutes after onset of hypoxia there was no significant difference (P =

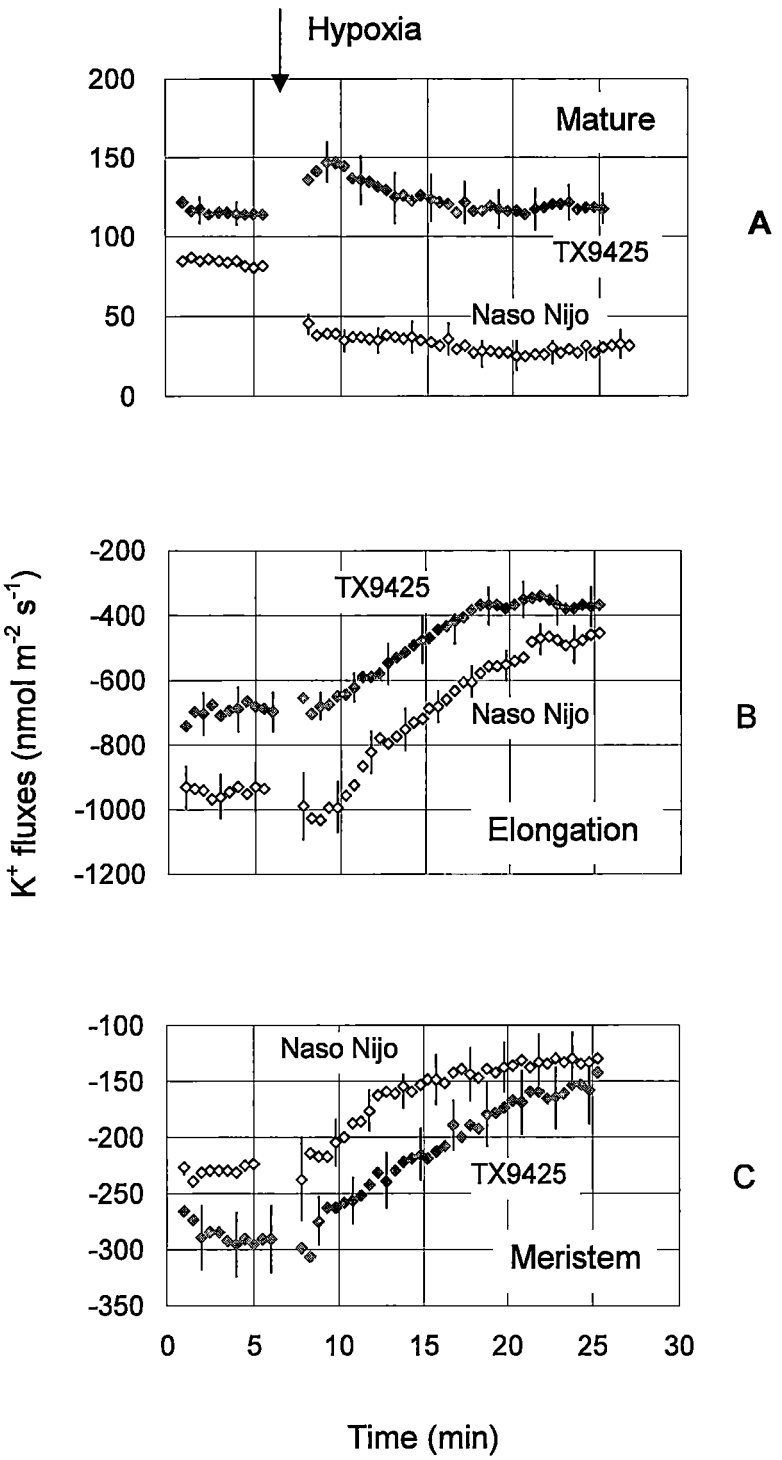

Fig. 5.6. Hypoxia-induced transient K^+ fluxes measured from mature (10mm from the root tip) (A), elongation (1mm) (B) and meristematic (0.3mm) (C) zones of Naso Nijo (open symbols) and TX9425 (closed symbols) roots. Error bars are SE (n =8).

0.05) in the steady-state K^+ fluxes between the two cultivars in either of those apical root zones. However, the overall magnitude of the reduction in K^+ efflux was significantly (P = 0.05) larger in Naso Nijo than in TX9425 when measured in the elongation zone (480 ± 72 and 330 ± 26 nmol m^{-2} s^{-1}, respectively; Fig. 5.6B).

5.4.2.2.2. K^+ fluxes from intact or decapped roots

The observed massive K^+ efflux from the elongation zone of both cultivars (Fig. 5.6B) was rather unexpected. One would assume that rapidly elongating roots (see Table 5.1) would require some substantial K^+ influx to increase cell turgor and drive the expansion growth. Indeed, such K^+ influx has previously observed in other barley cultivars (Chen *et al.* 2005). That was not the case in this study (Fig. 5.6B), where K^+ flux patterns were more similar to those reported by Wherrett *et al.* (2005) for wheat. The observed K^+ efflux was not attributed to growth conditions of plants, as qualitatively similar results were obtained for plants grown in (1) aerated solution as described in this chapter; (2) on wet paper rolls; (3) on the solid agar surface (in vertically-oriented Petri dishes); (4) under different light regimes (light *vs* dark grown); and (5) in the range of K^+ concentrations (from 0.1 to 5 mM K^+). In all cases, steady state K^+ efflux was measured from the elongation zone of horizontally placed roots (between -250 and -700 nmol m^{-2} s^{-1}). Therefore, it appears that some other confounding factors might contribute to the steady state fluxes in the elongation zone of barley roots in this study. One of these factors might be root gravitropism. Because of the technical difficulty to keep roots vertically, roots were placed horizontally for the convenience of measurement.

The standard procedure to avoid confounding effects of gravitropism is the use of decapped roots (Bjorkman and Cleland 1991). Accordingly, the root cap was gently removed from plant roots as described in the Section 5.3.2. When this was done, no net K^+ efflux was measured from the elongation zone of either cultivar used in this study (Fig. 5.7A), with steady state K^+ fluxes ranging between near zero in Naso Nijo to ~ 20 nmol m^{-2} s^{-1} K^+ influx in TX9425 (Fig. 5.7A). Hypoxia treatment

caused substantial increase in net K^+ uptake in the elongation zone of decapped roots in both cultivars (Fig. 5.7A) which is consistent with the observed hypoxia-induced decrease in net K^+ efflux in intact roots (Fig. 5.6B).

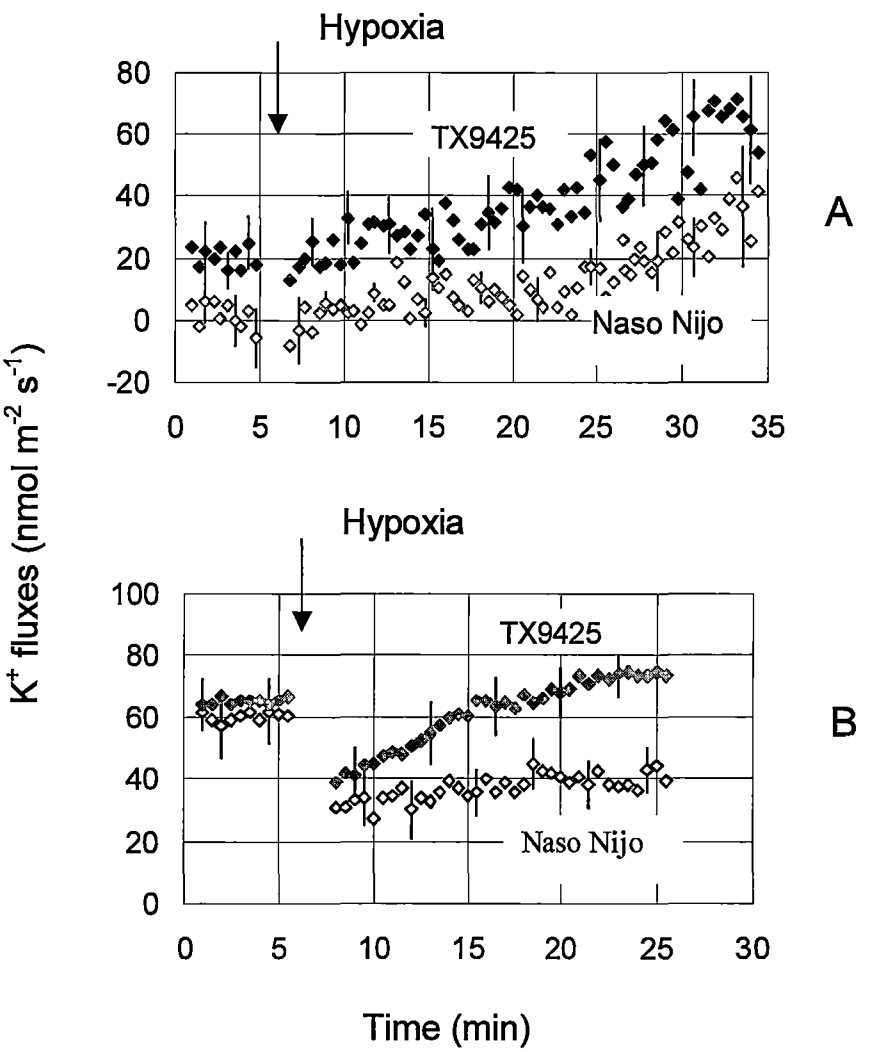

Fig. 5.7. Hypoxia-induced transient K^+ fluxes measured from (A) the elongation zone of decapped roots and (B) mature zone of excised roots of two barley cultivars: Naso Nijo (open symbols) and TX9425 (closed symbols). Error bars are SE (n =6 to 8).

In order to eliminate the possibility of internal oxygen transport and its impact on net ion fluxes from barley roots, excised roots were used in some experiments. Fig.

5.7B shows results of K^+ flux measurements for the mature zone of the excised roots of two cultivars. Although steady-state K^+ influx was slightly lower in excised compared with intact roots (Fig. 5.7B and 5.6A, respectively), a qualitatively similar type of K^+ flux response to hypoxia was observed. Hypoxic treatment caused a very sharp decline in K^+ uptake in the waterlogging sensitive cultivar Naso Nijo, while net K^+ influx in the waterlogging tolerant TX9425 20 min after stress onset was not significantly different from control (normoxia) (Fig. 5.7B). Taken together, these results suggest that oxygen transport from coleoptiles to roots had no impact on hypoxia-induced kinetics of ion fluxes in roots in my experiments.

5.4.2.2.3. H^+ fluxes

In the mature zone, onset of hypoxia had only a minor and temporary effect on H^+ flux kinetics in both cultivars (Fig. 5.8A). Twenty mins after hypoxic treatment, steady-state H^+ flux values were not significantly (P=0.05) different from those measured under normoxic conditions. Also, no significant (P=0.05) genotypical difference was observed (Fig. 5.8A). In contrast, in both the elongation and meristem regions, onset of hypoxia significantly (P = 0.05) reduced net H^+ influx measured in the root apex for both cultivars (Fig. 5.8B, C). In both these zones, significantly (P=0.05) greater reduction in net H^+ uptake was found in Naso Nijo (sensitive) than in TX9425 (tolerant).

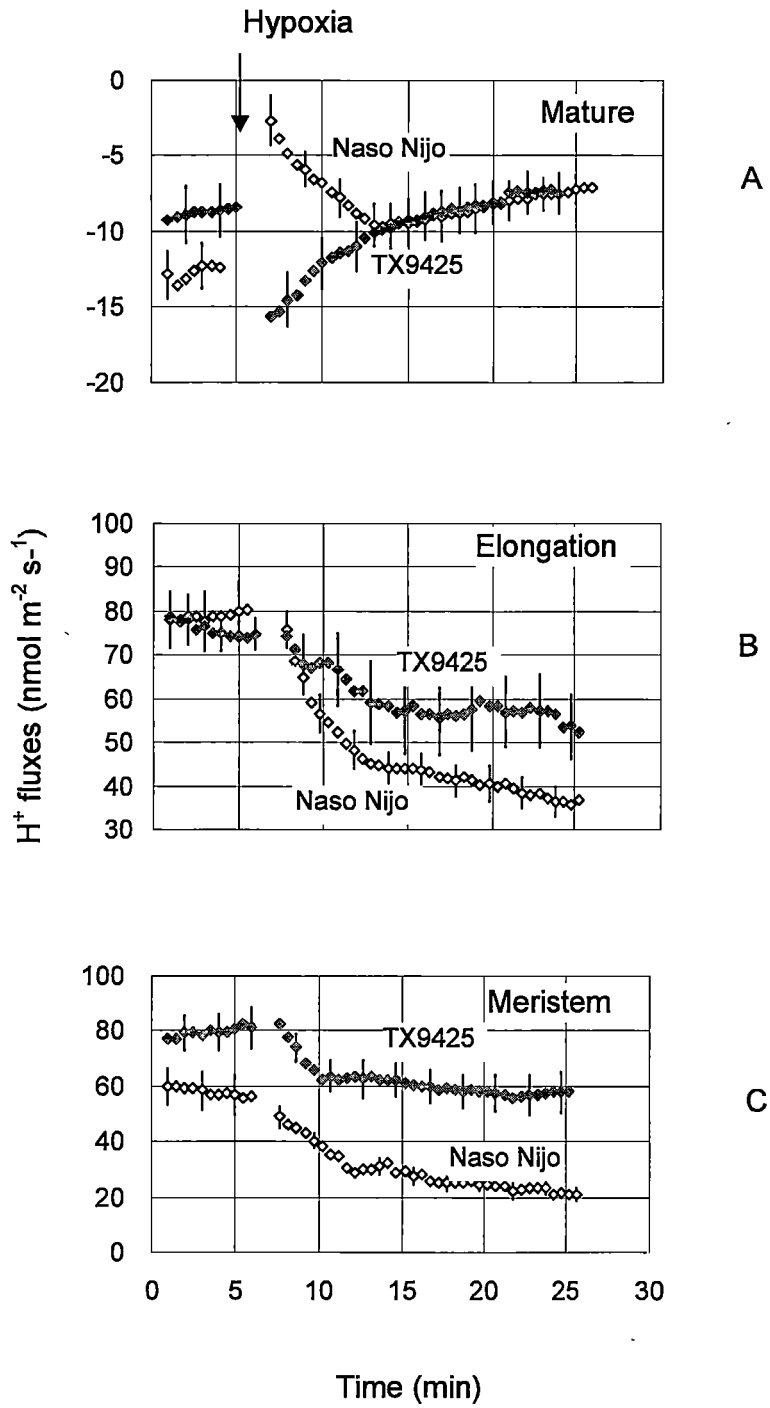

Fig. 5.8. Hypoxia-induced transient H^+ fluxes measured from mature (A), elongation (B) and meristematic (C) zone of Naso Nijo (open symbols) and TX9425 (closed symbols) roots. Error bars are SE (n =8).

5.4.3 Pharmacology

5.4.3.1. Effect of vanadate on hypoxia-induced ion flux kinetics

Root pre-treatment in 0.5 mM vanadate, a well-known inhibitor of plasma membrane H^+-ATPase, caused significant changes in both H^+ and K^+ flux patterns and their responses to hypoxia (Tables 5.2 and 5.3; Fig. 5.9).

H^+ fluxes. Much higher initial net H^+ influx was measured in both the elongation and mature zones in both cultivars under normoxia after vanadate pre-treatment (Table 5.2). Also, although pre-treatment with vanadate did not significantly affect the overall magnitude of H^+ flux response to hypoxia (Fig. 5.9C; Table 5.2), pre-treated roots showed a rapid increase in H^+ uptake (significant at $P = 0.05$) in the root elongation zone immediately after hypoxia treatment (Fig. 5.9C).

K^+ fluxes. Vanadate caused the initial K^+ efflux in the elongation zone to halve in both cultivars, as well as significantly ($P = 0.05$) reducing the magnitude of the K^+ flux responses to hypoxia (Table 5.3; Fig. 5.9A). In the mature zone vanadate pre-treatment resulted in initial K^+ fluxes being reversed from net uptake to K^+ efflux (Fig. 5.9B; Table 5.3). The magnitude of the hypoxia-induced shift towards large K^+ efflux was also severely reduced, or completely prevented, in the mature zone of vanadate pre-treated roots (Fig. 5.9B).

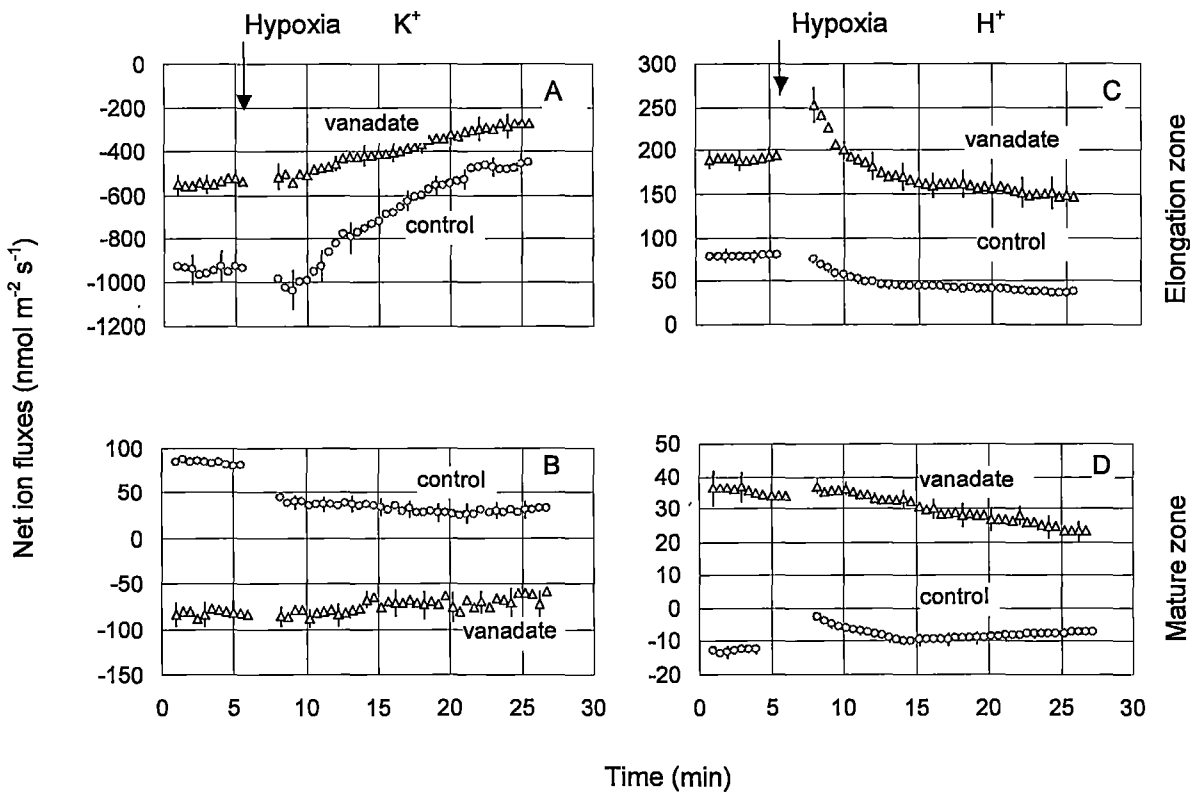

Fig. 5.9. Effects of vanadate pre-treatment on hypoxia-induced ion flux responses in Naso Nijo roots. Net K^+ (A, B) and H^+ (C, D) flux response were measured in elongation (A, C) and mature (B, D) zones of vanadate pre-treated roots (triangles). Open circles show hypoxia-induced responses from control (no vanadate) roots. Error bars are SE (n =8).

Table 5.2. Effect of membrane-transport inhibitors on quantitative characteristics of H^+ fluxes measured from the elongation and mature regions of roots of two barley cultivars before and after (20 min) onset of hypoxia. Statistics are means ± SE (n = 8).

Cultivar	*Zone*	*H^+ fluxes*			
		control	vanadate	TEA^+	Gd^{3+}
		Initial flux values (nmol m^{-2} s^{-1})			
Naso Nijo	Elong	80 ± 6.7	190 ± 8.2 ^{c}	55 ± 1.4 ^{b}	180 ± 16.9 ^{b}
	Mature	-12 ± 1.4	34 ± 3.1 ^{c}	-12 ± 0.8 ^{ns}	-9 ± 1.7 ^{ns}
TX9425	Elong	74 ± 3.1	160 ± 12.2 ^{c}	55 ± 4.6 ^{b}	196 ± 8.0 ^{c}
	Mature	-8 ± 1.6	14 ± 0.9 ^{c}	-1 ± 1.5 ^{a}	-7 ± 1.5 ^{ns}
		Magnitude of response (nmol m^{-2} s^{-1})			
Naso Nijo	Elong	-43 ± 7.1	-43 ± 3.1 ^{ns}	-25 ± 2.7 ^{a}	-40 ± 2.3 ^{ns}
	Mature	5 ± 1.6	-11 ± 2.1 ^{c}	6 ± 1.5 ^{ns}	2 ± 1.5 ^{ns}
TX9425	Elong	-22 ± 3.5	-50 ± 10.3 ^{a}	-21 ± 4.3 ^{ns}	-81 ± 9.5 ^{c}
	Mature	1 ± 1.5	-7 ± 2.0 ^{ns}	-2 ± 0.9 ^{ns}	1 ± 1.2 ^{ns}
		Steady state fluxes (at the end of transient) (nmol m^{-2} s^{-1})			
Naso Nijo	Elong	37 ± 3.3	147 ± 4.0 ^{c}	30 ± 2.3 ^{ns}	140 ± 14.1 ^{b}
	Mature	-7 ± 0.8	23 ± 1.9 ^{c}	-6 ± 1.0 ^{ns}	-7 ± 0.6 ^{ns}
TX9425	Elong	52 ± 5.9	110 ± 14.3 ^{a}	34 ± 2.3 ^{ns}	115 ± 14.3 ^{a}
	Mature	-7 ± 1.4	7 ± 1.7 ^{c}	-3 ± 1.7 ^{ns}	-6 ± 0.5 ^{ns}

$^{a, b, c}$ Significant compared with control at P = 0.05, 0.01 and 0.001, respectively (by Student's *t* test); ^{ns} Not significant.

Table 5.3. Effect of membrane-transport inhibitors on quantitative characteristics of K^+ fluxes measured from the elongation and mature regions of roots of two barley cultivars before and after (20 min) onset of hypoxia. Statistics are means ± SE (n = 8).

Cultivar	*Zone*	*K^+ fluxes*			
		control	vanadate	TEA^+	Gd^{3+}
		Initial flux values (nmol m^{-2} s^{-1})			
Naso Nijo	Elong	-930 ± 68.4	-540 ± 42.6 ^{c}	-475 ± 40.3 ^{c}	-634 ± 39.2 ^{b}
	Mature	80 ± 2.7	-84 ± 10.7 ^{c}	-11 ± 1.1 ^{c}	26 ± 4.1 ^{c}
TX9425	Elong	-700 ± 58.1	-340 ± 6.9 ^{c}	-230 ± 32.0 ^{c}	1190 ± 87.7 ^{c}
	Mature	114 ± 6.7	-75 ± 4.4 ^{c}	12 ± 2.6 ^{c}	-40 ± 4.1 ^{c}
		Magnitude of response (nmol m^{-2} s^{-1})			
Naso Nijo	Elong	480 ± 72.8	270 ± 46.6 ^{a}	180 ± 23.0 ^{b}	139 ± 39.4 ^{b}
	Mature	-48 ± 9.6	24 ± 6.0 ^{c}	-1 ± 1.6 ^{b}	-51 ± 6.5 ^{ns}
TX9425	Elong	330 ± 25.8	120 ± 23.2 ^{c}	152 ± 29.8 ^{c}	-505 ± 84.1 ^{c}
	Mature	4 ± 1.3	50 ± 5.6 ^{b}	-12 ± 2.0 ^{ns}	10 ± 1.1 ^{ns}
		Steady state fluxes (at the end of transient) (nmol m^{-2} s^{-1})			
Naso Nijo	Elong	-450 ± 51.1	-270 ± 51.1 ^{a}	-295 ± 40.9 ^{a}	-495 ± 31.2 ^{ns}
	Mature	32 ± 9.2	-60 ± 7.8 ^{c}	-12 ± 2.5 ^{b}	-25 ± 4.2 ^{b}
TX9425	Elong	-370 ± 57.5	-220 ± 31.7 ^{a}	-78 ± 10.5 ^{c}	685 ± 94.6 ^{c}
	Mature	118 ± 9.5	-25 ± 5.6 ^{c}	0 ± 1.9 ^{c}	-30 ± 2.9 ^{c}

$^{a, b, c}$ Significant compared with control at P = 0.05, 0.01 and 0.001, respectively (by Student's *t* test); ^{ns} Not significant.

5.4.3.2. Effect of TEA on hypoxia-induced ion flux kinetics

TEA is a known inhibitor of K^+ selective channels (Maathuis and Sanders 1996). In the elongation zone, 20 mM TEA pre-treatment caused significant (P = 0.05) 2-3 fold reduction both of initial K^+ efflux and of K^+ flux response to hypoxia (Table 5.3; Fig. 5.10A). Both trends were observed in WL-sensitive and WL-tolerant cultivars. In the mature zone, the TEA pre-treatment caused a significant (P = 0.001) shift (~ 100 nmol m^{-2} s^{-1}) towards K^+ efflux in both cultivars and almost completely blocked K^+ flux responses to hypoxia (Fig. 5.10B; Table 5.3). TEA pre-treatment of Naso Nijo also reduced net H^+ uptake in the elongation zone and nearly halved the hypoxia-induced H^+ fluxes in this zone (Fig. 5.10C).

5.4.3.3. Effect of Gd^{3+} on hypoxia-induced ion flux kinetics

Gd^{3+} is a known blocker of non-selective cation channels, which are known to be K^+-permeable (Demidchik *et al.* 2002). Significant (P = 0.01) changes in steady-state K^+ flux levels were observed in Gd^{3+} pre-treated roots in both zones under normoxic conditions (Table 5.3; Fig. 5.11A, B). Also, a significant change in hypoxia-induced K^+ flux was observed in the elongation zone of both cultivars, but not in the mature zone (Fig. 5.11A, B; significant at P = 0.05). No clear patterns of Gd^{3+} effect on H^+ flux were found (Fig. 5.11 C, D).

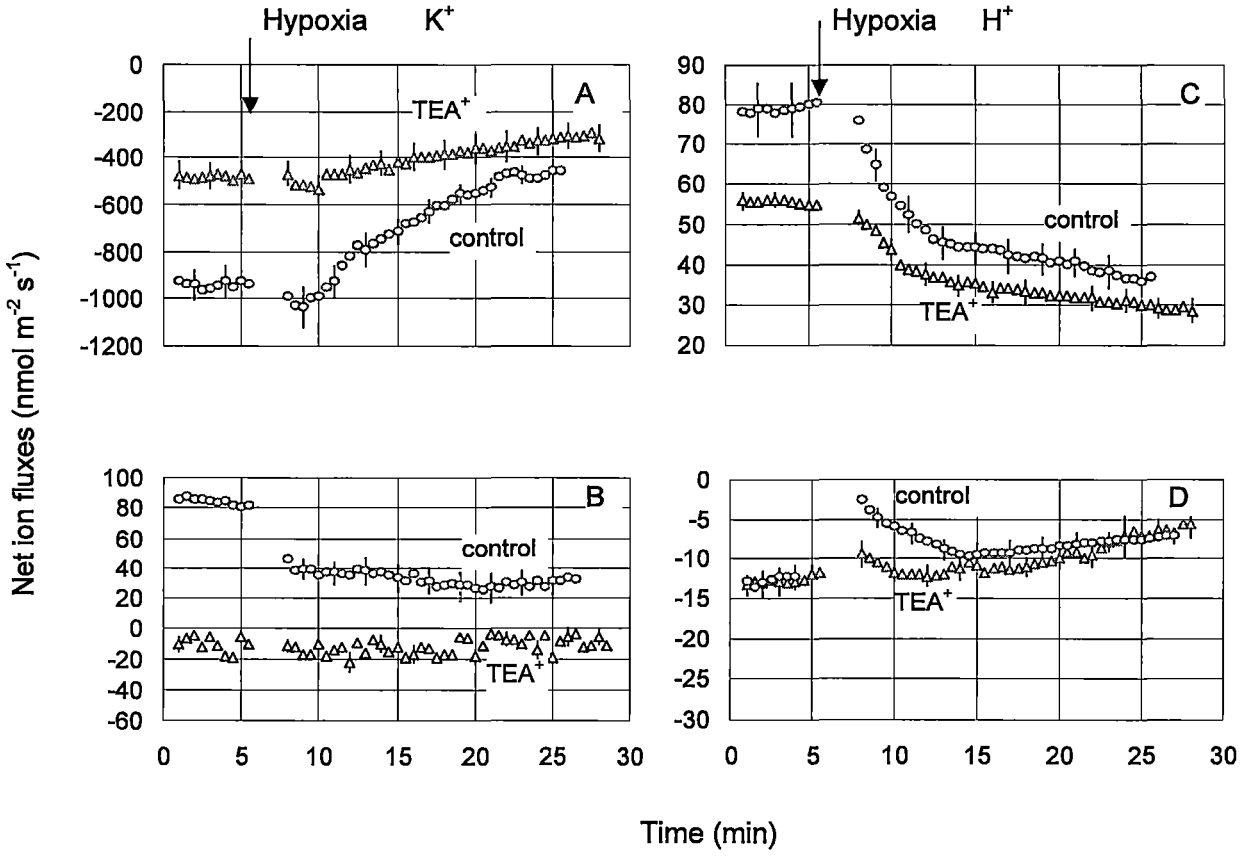

Fig. 5.10. Effects of TEA pre-treatment on hypoxia-induced ion flux responses in Naso Nijo roots. Net K^+ (A, B) and H^+ (C, D) flux response were measured in elongation (A, C) and mature (B, D) zones of TEA pre-treated roots (triangles). Open circles show hypoxia-induced responses from control (no TEA) roots. Error bars are SE (n =8).

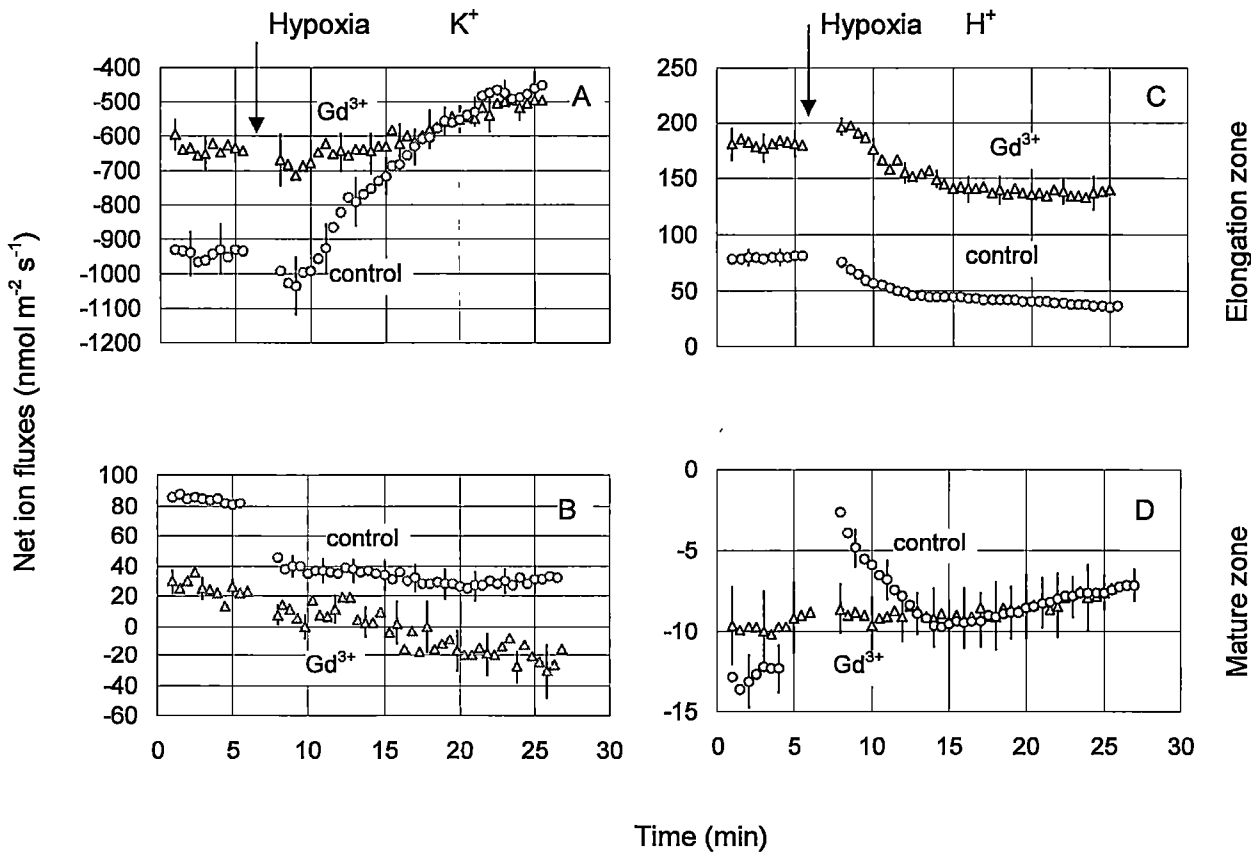

Fig. 5.11. Effects of Gd^{3+} pre-treatment on hypoxia-induced ion flux responses in Naso Nijo roots. Net K^+ (A, B) and H^+ (C, D) flux response were measured in elongation (A, C) and mature (B, D) zones of Gd^{3+} pre-treated roots (triangles). Open circles show hypoxia-induced responses from control (no Gd^{3+}) roots. Error bars are SE (n = 8)

5.5. DISCUSSION

So far, there have been only a few reports of O_2 flux measurement near plant roots (Bidel *et al.* 2000, Mancuso and Boselli 2002, Mancuso *et al.* 2000); none of them correlated O_2 profiles with fluxes of specific ions under hypoxia conditions. The only similar studies were undertaken in algal cells, where O_2 and H^+ fluxes were "matched" (Porterfield and Smith 2000, Serikawa *et al.* 2000). In this study, I combined the advantages of microelectrode ion and O_2 flux measurements to "map" the root surface and investigate effect of hypoxia on ion flux profile along the barley roots in an attempt to reveal physiological mechanisms underlying plant tolerance to waterlogging.

5.5.1 Feasibility of MIFE measurements in hypoxic conditions

Over the last 15 years, non-invasive ion flux measurements have been widely used to address plant responses to various types of abiotic stresses such as salinity (Babourina *et al.* 2000, Shabala 2000, Shabala *et al.* 2003), pH (Babourina *et al.* 2001, Shabala *et al.* 1997), osmotic stress (Shabala *et al.* 2000, Shabala and Lew 2002, Shabala and Newman 1998), chilling (Shabala and Shabala 2002, Shabala and Newman 1997a), wounding (Hush *et al.* 1992, Meyer and Weisenseel 1997), Al^{3+} toxicity (Ryan and Kochian 1993, Ryan *et al.* 1992), and oxidative stress (Demidchik *et al.* 2003). In all these papers measurements have been made in "conventional" solutions, having known ion mobility.

To impose controlled hypoxic stress onto hydroponically growing roots, two options have been used. In the first option, N_2 is constantly bubbled through the measuring chamber (Visser *et al.* 1996c, Xia and Roberts 1996). Such a method is not appropriate however for flux measurements, which require "unstirred layer" conditions (Newman 2001). The second option is to use a weak agar solution as the root growth medium to diminish slow convective O_2 transfer from the surface (Armstrong *et al.* 2000, Wiengweera *et al.* 1997). It was unclear, however, to what

extent the presence of agar in solution would affect selectivity and other characteristics of ion-selective LIX and, thus, whether the MIFE measurements would be feasible under such conditions.

I addressed these issues. My results showed the absence of any significant (P = 0.05) effect of 0.05% agar (concentration used in this study) on LIX characteristics (Fig. 5.5A). I have also provided evidence that there was no significant difference between plant responses to either type (e.g. N_2/oil and agar) of hypoxia treatment, either in terms of O_2 concentration changes in the bath or in the ion flux responses from roots (Fig. 5.5B, C). Taken together, the data validated the use of the MIFE technique to measure net ion fluxes in agar-enriched solution.

5.5.2 Microelectrode O_2 flux measurements resolved different O_2 requirements for functionally different root zones in barley

In my experiments, O_2 uptake rate in the root apex was twice that in the rest of the root (mature zone) (Fig. 5.2A). The highest O_2 uptake was measured in the elongation zone, 1 mm from the root tip (Fig. 5.2A), consistent with results of Mancuso & Boselli (2002) on roots of three *Vitis* genotypes. The large O_2 demand of the functionally more active root apex is in accord with results of Reid *et al.* (1985a) who showed that both ATP content and the respiration rate in the tip region (0-10mm) were double those of the rest of the barley root. The absolute values of net O_2 fluxes under normoxic conditions in my experiment (1300-1400 nmol m^{-2} s^{-1} and 600-700 nmol m^{-2} s^{-1} for elongation and mature zones, respectively), are also comparable to the values reported by Mancuso & Boselli (2002).

Hypoxia treatment rapidly depleted O_2 concentration in the root medium (Fig. 5.3). As a result, O_2 influx was also significantly reduced along the whole root (Fig. 5.4). The effect was time dependent (Fig. 5.2B), with O_2 influx progressively decreasing as hypoxia stress developed. After 5h of hypoxia, the mature zone at 5 cm from the root tip showed net O_2 efflux (O_2 losses). Functionally active tissues in the root

apex still exhibited net O_2 uptake, although at severely reduced rates (Fig. 5.2B). This is in accord with net O_2 efflux observed in three *Vitis* species after 4-5 h anaerobic treatment (Mancuso and Boselli 2002). This net O_2 efflux is usually termed radial oxygen loss (ROL) and has been reported in many species (Colmer 2003b).

5.5.3 Effect of the root cap removal

As K^+ is critical for the root expansion growth, a substantial K^+ efflux from the elongation zone of intact roots (Fig 5.6B) was rather unexpected and indicated that roots were not in a steady state after being immobilised horizontally in the chamber and put onto the microscope stage. The only possible way to reach steady state conditions was to delay commencing measurements, keeping plants longer in the measuring chamber (for several hours at least). This was out of the question as, after several hours in the horizontal position, root tips were gravitropically bent and microelectrode access to the elongation zone was not possible. The only way to avoid the confounding effect of gravitropism was gently to remove the root cap, making roots gravity insensitive (Bjorkman and Cleland 1991). When this had been done, no net K^+ efflux was measured from the elongation zone of either cultivar used in this study (Fig. 5.7A). Interestingly, the previous measurements on barley (Chen *et al.* 2005) did not encounter such a problem, and small but substantial K^+ influx (similar to that shown in Fig. 5.7A) was measured one hour after root immobilisation. The difference may be attributable to genotypic-specific features of gravitropic responses of cultivars used in these two studies.

Contribution of other factors also cannot be completely ruled out, as the root cap is known to be an important perception centre for not only gravity, but also some other stimuli. One of them is light. Wang *et al.* (2002) reported that rice roots lost their negative phototropism after the root cap was removed, and resumed it again when the new cap was re-grown. As roots were exposed to microscope light during ion flux measurements in my experiments, different phototropic sensitivity between

cultivars may also explain the difference between net K^+ fluxes in the elongation zone of barley roots in this study and the previous results from our laboratory (Chen *et al.* 2005). Cross-talk between gravi- and photo- perception by roots is also possible (Galen *et al.* 2004, Vitha *et al.* 2000). Specific details of this interaction and differential sensitivity between cultivars are clearly beyond the scope of this paper and should be the subject of a separate investigation.

5.5.4 Root apex and mature zone exhibit qualitatively different kinetics of K^+ flux responses to hypoxia

Buwalda *et al.* (1988) suggested that the K^+ loss observed during the early stages of hypoxia was due to membrane depolarization rather than to increases in the permeability of membranes to K^+. The depolarization was caused presumably by decreased ATP synthesis, as membrane potentials were closely correlated with ATP concentrations in barley roots exposed to sodium azide (Reid *et al.* 1985a). It remained to be answered however whether this effect is uniform along the whole root, or whether some tissues/zones are more sensitive than others to O_2 deprivation.

In full agreement with previous reports (Buwalda *et al.* 1988, Reid *et al.* 1985a), hypoxia significantly inhibits K^+ uptake in the mature zone of the sensitive Naso Nijo cultivar but not of the tolerant TX9245 (Fig. 5.6A). However, in elongation and meristem zones the hypoxia reduced, not increased, K^+ efflux from the root (Fig. 5.6 B, C), retaining more K^+ in oxygen-stressed roots. This is further supported by experiments on decapped roots, showing increased net K^+ uptake in response to hypoxia in both cultivars (Fig. 5.7A). These results are in line with the hypothesis of Greenway & Gibbs (2003) who proposed that in anoxia-tolerant tissues, energy flow during anoxia must be directed towards essential nutrient transport, especially in such a sensitive part as the root apex.

My findings are also consistent with those reported by Colmer *et al.* (2001) showing that membrane permeability of rice coleoptile tissue (which is known to be anoxia-tolerant) to K^+ was reduced ~ 17-fold in anoxic conditions. Further supporting evidence may be found in animal literature. For certain animal cells exposed to O_2 concentrations at 45% of aerated levels, a large decrease in the probability of opening of the outward-rectifying K^+ channels has been measured using the patch clamp technique (Lopez-Barneo 1994). Reggiani (1997) also advocated cAMP-triggered KOR closure under anoxia.

5.5.5 H^+-ATPase is likely to mediate hypoxia-induced H^+ fluxes in barley roots

As reduction in O_2 supply leads to up to 97% reduction in the rate of energy production (Greenway and Gibbs 2003), plant roots have to compensate for this loss by accelerating sugar metabolism and glycolysis. Decrease in cytosolic ATP is also the main cause of the rapid acidification of the cytosol. Such acidification may result both from inhibition of proton pumping by low ATP concentration and from proton release through ATP hydrolysis (Ricard *et al.* 1994). The H^+-ATPase is highly sensitive to cytosolic pH (Slayman 1987), so increased H^+ extrusion is expected to counter the acidosis induced by hypoxia as long as the pool of ATP is not limiting (Gout *et al.* 2001). According to my results, hypoxia shifted net H^+ fluxes towards efflux in elongation and meristematic regions (Fig. 5.8B, C). This may be interpreted as an up-regulation of H^+ pumping in order to counter the hypoxia-induced cytosol acidification. No significant changes in net H^+ fluxes were found in the mature zone (Fig. 5.8A), presumably due to the lack of O_2 influx (Fig. 5.2) required to drive this process. Pre-treatment with vanadate, a known inhibitor of plasma membrane (PM) H^+-ATPase, significantly increased net H^+ influx in both elongation and mature root zones (Fig. 5.8C, D; Table 5.2), strongly supporting the hypothesis that a substantial component of the measured H^+ transients was due to changes in activity of PM H^+- ATPase.

5.5.6 Several K^+ -transporting systems are likely to mediate root responses to hypoxia

Several lines of evidence suggest that both specific K^+-permeable channels and non-selective cation channels (NSCC) mediate barley root responses to hypoxia. First, TEA, a known K^+ channel blocker, strongly affected initial K^+ flux values (Table 5.3) as well as depressing (in elongation zone; Fig. 5.10A) or even preventing (in mature zone; Fig. 5.10B) K^+ flux responses to hypoxia. In the mature zone, TEA caused a significant ($P = 0.05$) shift towards net K^+ efflux under normoxia (Fig. 5.10B), while in the elongation zone the opposite trend was observed (~ 50% reduction in net K^+ efflux). A plausible hypothesis explaining these findings is that K^+ exchange in barley roots is mediated predominantly by K^+ - inward- (KIR) and outward- (KOR) rectifying channels, respectively in the elongation and mature zones. Both of these channels are known to be TEA sensitive (Maathuis and Sanders 1996). Importantly, both are also voltage dependent (Cherel 2004). This may explain why net K^+ fluxes were affected by root pre-treatment with 0.5 mM vanadate (Table 5.3; Fig. 5.9A, B). Proton pumps are the primary motors that build up transmembrane electrochemical proton gradients, which drive transport of ions as well as a variety of uncharged molecules (Felle 2001). Inhibition of H^+-ATPase activity by vanadate was expected to cause plasma membrane depolarization and lead to the observed changes in K^+ fluxes through voltage-dependent K^+ channels.

It also appears that at least part of the K^+ flux is mediated by NSCC. Root pre-treatment with 30 μM Gd^{3+} had a significant ($P = 0.05$) effect on K^+ flux patterns in both zones (Fig. 5.11A, B). In the elongation zone, Gd^{3+} caused significant change in initial K^+ flux as well as significant change in the magnitude of hypoxia-induced response in both cultivars (Table 5.3). In contrast, in the mature zone the magnitude of hypoxia-induced K^+ flux response was not significantly ($P = 0.05$) affected by Gd^{3+} treatment, despite substantial shift in initial K^+ flux values. Overall, my results

are consistent with the scenario that hypoxia-induced K^+ flux responses are mediated by both KIR and NSCC channels in the elongation zone, while in the mature zone KOR channels are likely to be the key players. Further experiments using genetically modified material (e.g. *Arabidopsis* K^+ transport mutants) are needed to fully address this issue.

5.5.7 Genotypic differences

My previous work (Chapter 4 and Pang *et al.* 2004) showed that the barley cultivars employed in this study (TX9425 and Naso Nijo) differ strongly in their waterlogging tolerance, being WL-tolerant and WL-sensitive, respectively. It was also shown that this difference in WL tolerance could be at least partly attributed to a significant difference in the pattern of aerenchyma formation (Chapter 4 and Pang *et al.* 2004). However, the formation of aerenchyma is a lengthy process requiring several days. Are there more rapid mechanisms underlying the difference in waterlogging tolerance between these cultivars?

Under normoxia, the O_2 concentrations around the elongation and mature zones were about 190 and 150 μmol/L, respectively, in both cultivars (Fig 5.4A). Twenty mins after hypoxia, there was no significant difference between the cultivars in the root elongation zone. In the mature zone however, much higher O_2 influx was measured for TX9425 than for Naso Nijo (Fig. 5.4B) (significant at P=0.05). Mancuso and Boselli (2002) found that, in the root meristem, tolerant *Vitis* species decreased O_2 influx much slower than sensitive species, under hypoxia stress. These authors suggested less respiratory demand and/or better internal transport from the shoots in tolerant varieties. However, in my experiments no significant difference was found in root ion flux responses to hypoxia when intact seedlings were compared with excised roots (Fig. 5.6A and 5.7B, respectively). Thus, oxygen transport from shoot to root is not likely to be a significant contributor to waterlogging tolerance in this study.

A higher level of ATP is considered to be a significant contributing factor to subsequent plant survival under anoxic stress (Kato-Noguchi 2000). From this point of view, the higher O_2 uptake rate in the mature zone of tolerant TX9425 would permit the maintenance of ATP to preclude the adverse effects of hypoxia on cytosolic metabolism. Overall, this may result in the ability of tolerant TX9425 to maintain relatively stable K^+ uptake, while the sensitive Naso Nijo showed significant ($P = 0.05$) decline in net K^+ influx in mature zone in response to hypoxia (Fig. 5.6A). This might affect root K^+ nutrient status and thus the overall plant performance under hypoxia, keeping in mind a plethora of roles K^+ plays in plant metabolism (Leigh 2001).

Overall, it appears that oxygen deprivation has an immediate and substantial effect on root ion flux patterns, and this effect is rather different in waterlogging sensitive and tolerant cultivars. It remains to be answered in future studies to what extent this difference in ion flux responses to hypoxia is a factor conferring waterlogging tolerance in barley, or whether it is merely a consequence of the difference in some other physiological or anatomical (Chapter 4 and Pang *et al.* 2004) mechanisms mediating plants adaptive responses to waterlogging.

Chapter 6. Effect of secondary metabolites associated with anaerobic soil conditions on ion fluxes and electrophysiology in barley roots

6.1. Abstract

The effects of secondary metabolites produced by waterlogged soils on net K^+, H^+ and Ca^{2+} fluxes were studied using the non-invasive MIFE® system in the mature zone of a barley (*Hordeum vulgare* L.) cultivar Naso Nijo. My measurement revealed that all three lower monocarboxylic acids (formic, acetic and propionic acids) and three phenolic acids (benzoic, 2-hydroxybenzoic, 4-hydroxybenzoic acids) caused immediate net influx of H^+ and the reduction of K^+ uptake, while Mn^{2+} treatment caused K^+ to quickly return to the initial level following the net efflux in the first few minutes and gradual increase of H^+ influx. Phenolic acids slightly increased the influx of Ca^{2+} immediate after treatment, but not in other chemicals. Plant roots showed different responses of ion fluxes and membrane potential to these chemicals in the long term (24 h). 24 h treatment with all chemicals significantly reduced the K uptake, and the adverse effects of phenolic acids were smaller than with monocarboxylic acids and Mn^{2+}. Treatment with monocarboxylic acids for 24 h reversed H^+ from net efflux to net influx, while all three phenolic acids did not cause significant effects compared with the control. Phenolic acids caused significant net Ca^{2+} efflux from roots pre-treated for 24 h. The possible model explaining effects of secondary metabolites on membrane transport activity is suggested.

6.2. Introduction

Under waterlogging conditions, oxygen level is reduced rapidly in the soil solution by the respiration of soil microorganisms and plant roots, resulting in either hypoxia (oxygen depletion) or anoxia (the absence of molecular oxygen). Once molecular oxygen has been consumed in respiration, various populations of microorganisms utilize other terminal electron acceptors for respiration acceptors in a well-defined sequence of anaerobic reduction-oxidation processes, resulting in a further decrease of the soil redox potential (Blom and Voesenek 1996). As a result, significant accumulation of toxic substances owing to the anaerobic metabolism of plants or microbes occurs in waterlogged soil (Armstrong and Armstrong 2001, Lynch 1977, Tanaka *et al.* 1990). Materials potentially toxic to plants accumulated in flooded soils include reduced manganese, iron, hydrogen sulphide, various organic acids, and ethylene (Armstrong and Gaynard 1976).

The type and amount of organic acid produced depends upon the fermentive character of the microflora, the kind and amount of organic materials added, and on the prevailing soil conditions (Rao and Mikkelsen 1977). Lynch *et al.* (1980) identified various organic acids including phenylacetic, cinnamic, p-hydroxyphenylpropionic, p-coumaric, and 3,4-dihydroxyphenyl propionic acids as products of the anaerobic decomposition of couch grass rhizomes. Tanaka *et al.* (1990) found that rice root growth was inhibited by micromolar ($2*10^{-4}$ M) concentrations of phenolic acids such as benzoic, 2-hydroxybenzoic, 2-phenylpropionic, and 3-phenylpropionic acids formed in flooded soils amended with wheat straw both in the laboratory and in the field. These authors also suggested that under natural conditions of waterlogging, the amount of accumulated phenolic acids might be much higher. Experiments by Lynch (1978) showed that acetic acid could be accumulated at concentrations of 15 mM in the soil-straw slurries within 6 days under laboratory conditions and suggested that it could be even higher in the field. In another study, concentration of propionic acid in water

from around the rotting underground parts of Phragmites rhizomes was found to be around 10 mM, while acetic acid reached ~35 mM level (Armstrong and Armstrong 1999).

Also increased is accumulation of toxic micronutrients in waterlogged plants. Clark *et al.* (1957) found that Mn content of rice plants grown under submerged conditions was tenfold greater than that found in plants grown without submergence. Ashraf and Rehman (1999) reported that Fe and Mn contents increased 80- and 20-fold (to 390 and 148 mg kg^{-1}, respectively) in sandy loam soil as a result of 34 d flooding. The same order of magnitude increase was also reported by Stieger and Feller (1994).

In most temperate conditions the production of phytotoxins by micro-organisms from straw residues has been implicated as the prime cause of the detrimental effect on crop yield under anaerobic conditions (Cochran *et al.* 1977). Root elongation of rice seedlings was decreased by increased organic acid concentration (Rao and Mikkelsen 1977); Lynch (1977) showed that millimolar (7 to 15 mM) concentrations of acetic acid significantly retarded the extension of barley roots. Consistent results were reported by Robinson and Taylor (1974) who showed that acetic acid at 0.33 to 14.3 mM inhibited the respiration of oat root tips, reducing it to 50% at the maximum concentration.

It is not clear to what extent accumulation of toxic metabolites is causally linked to observed deficiency of major macronutrients in waterlogged soils. Energy deficiency caused by lack of O_2 reduced the availability of many essential nutrients including nitrogen, phosphorus, sulphur and most of the trace elements under waterlogging conditions (Drew 1988). In addition, there are reports suggesting that inorganic nutrient uptake may be also impaired by accumulated organic acids (Mitsui *et al.* 1954, Rao and Mikkelsen 1977). Mechanisms of such impairment remain to be investigated.

So far, most reports dealt with analysis of the overall changes in ion content in plant tissues, or with monitoring kinetics of nutrient depletion in growth solution (Glass 1973, Glass 1974b, Jackson and St. John 1980). Due to methodological limitations (relatively poor spatial and temporal resolution), these experiments failed to provide answers about the specific ionic mechanisms involved. The above methodological issues may be successfully overcome when using microelectrode ion flux measuring (the MIFE) technique (Shabala 2006, Shabala 2003). The non-invasive MIFE® system (University of Tasmania, Hobart, Australia) has very high spatial (few μm) and temporal (several seconds) resolution and has been successfully applied to the measurement of ion flux kinetics under a cultivar of stress conditions (Shabala *et al.* 2003, Shabala and Lew 2002, Shabala and Newman 1997a). In this study, this technique was used to quantify both the immediate responses of ion fluxes and long-term (after 24 h treatment) responses to secondary metabolites associated with anaerobic soils.

6.3. Materials and Methods

6.3.1 Plant material and growth conditions

Waterlogging sensitive (according to Chapter 4) barley (*Hordeum vulgare* L cv Naso Nijo) seedlings were grown hydroponically for 3 to 4 days on a floating mesh in plastic containers above 0.5 L of aerated nutrient solution containing 0.1mM $CaCl_2$ and 0.2 mM KCl under laboratory conditions (temperature + 24 °C; 16 h photoperiod; fluorescent lighting about 150 μmol m^{-2} s^{-1}) essentially as described by Chen *et al.* (2005). Seedlings were used for measurement when their root length was 60 to 80 mm.

6.3.2 Ion flux measurements

Net fluxes of H^+ and K^+ were measured using non-invasive microelectrode ion flux measuring (the MIFE) technique (University of Tasmania, Hobart, Australia). Details were described in Section 3.5 and chapter 5.

6.3.3 Experimental protocol

One hour before measurements, 5 mL basic solution (0.1mM $CaCl_2$, 0.2 mM KCl, pH 5.5 unbuffered) was added to a plexiglass measuring chamber (100 mm long, 30 mm deep, and 4 mm wide). A seedling was taken from the growth container and placed immediately into the chamber. The root was immobilised in the horizontal position by fine Teflon partitions 5 mm above the floor of the chamber as described in Chapter 5. The chamber was put onto the microscope stage in the Faraday cage and the plant was allowed to adapt to experimental conditions. Ion selective microelectrodes were positioned 50 μm above the root tissue in the mature zone (10 mm from the tip). During measurements electrodes moved vertically in a square-wave manner (10-s cycle; travel range 50 μm) driven by a hydraulics manipulator, details are described in Chapter 5.

In transient experiments, steady state fluxes were measured for five minutes. Then 5 ml of basic solution containing the double concentration of an appropriate chemical were added into the chamber, and measurements were continued for another 30 min. About 2 min were required for the unstirred layer conditions to be reached. This period of time was discarded from the analysis and appears as a gap in the figures.

For the measurement of long-term chemical effects on root ion fluxes and membrane potential, chemicals were added into the plastic growth container (basic solution) 24h before measurement. The final concentrations of phenolic acids (benzoic acid, 2-hydroxybenzoic acid and 4-hydroxybenzoic acid) were 200 μM, volatile monocarboxylic organic acids (formic acid, acetic acid and propionic acid) were 10 mM, Mn^{2+} was 300 mg L^{-1}. Solution pH was adjusted to 5.5 (using HCl/NaOH) in all treatments and checked frequently. Solutions were aerated continuously during 24-h period of treatment.

6.3.4 Membrane potential measurements

The roots of intact barley plants were mounted in a measuring chamber and the roots were gently secured in a horizontal position with small plastic blocks. Experimental conditions were the same as those for the ion flux measurement. The plant was allowed to stabilize for 60 mins. Measurements of the electrical potential difference (V_m) across the root-cell membranes were made in the root mature zone between 1-2 cm from the root tip. The borosilicate glass microelectrodes (Clark Electromedical Instruments, Reading, UK) were filled with 1 M KCl, connected to an electrometer via a Ag-AgCl half-cell and inserted into the root tissue with a manually operated micromanipulator (Narishige, Japan). The membrane potential value was calculated as the difference between the values in the solution initially and after impalement.

6.4. Results

6.4.1 Transient ion fluxes in response to secondary metabolites

6.4.1.1. K^+ fluxes

Net K^+ uptake of about 60 nmol m^{-2} s^{-1} was measured from mature epidermal root cells of 3-d old barley seedlings in control (steady state) conditions. Addition of phenolic compounds (benzoic acid, 2-hydroxybenzoic acid, 4-hydroxybenzoic acid; 200 μM working concentration) and volatile monocarboxylic organic compounds (formic acid, acetic acid, propionic acid; 10 mM working concentration) rapidly decreased net K^+ influx (Fig. 6.1). Among three different phenolic acids, 2-hydroxybenzoic acid and 4-hydroxybenzoic acid caused much more adverse effects on K^+ uptake compared with benzoic acid (Fig. 6.1A), completely arresting net K^+ uptake within 10 min after the treatment. All three volatile monocarboxylic organic acids not only arrested K^+ influx but caused significant ($P < 0.001$) K^+ efflux from barley roots, with an effect increasing in the following sequence: propionic acid >> acetic acid > formic acid (Fig. 6.1B). This effect was specific and not related to the

changes in osmolality of experimental solution, as isotonic treatment with KCl, NaCl or Na-gluconate caused no K^+ efflux from barley roots (data not shown). Adding 300 mg L^{-1} Mn^{2+} caused almost instantaneous reduction of K^+ uptake, which quickly (within 5 min) returned to its initial value (Fig. 6.1C). In general, the effect of monocarboxylic organic acids on root K^+ fluxes was significantly stronger than that caused by phenolic acids.

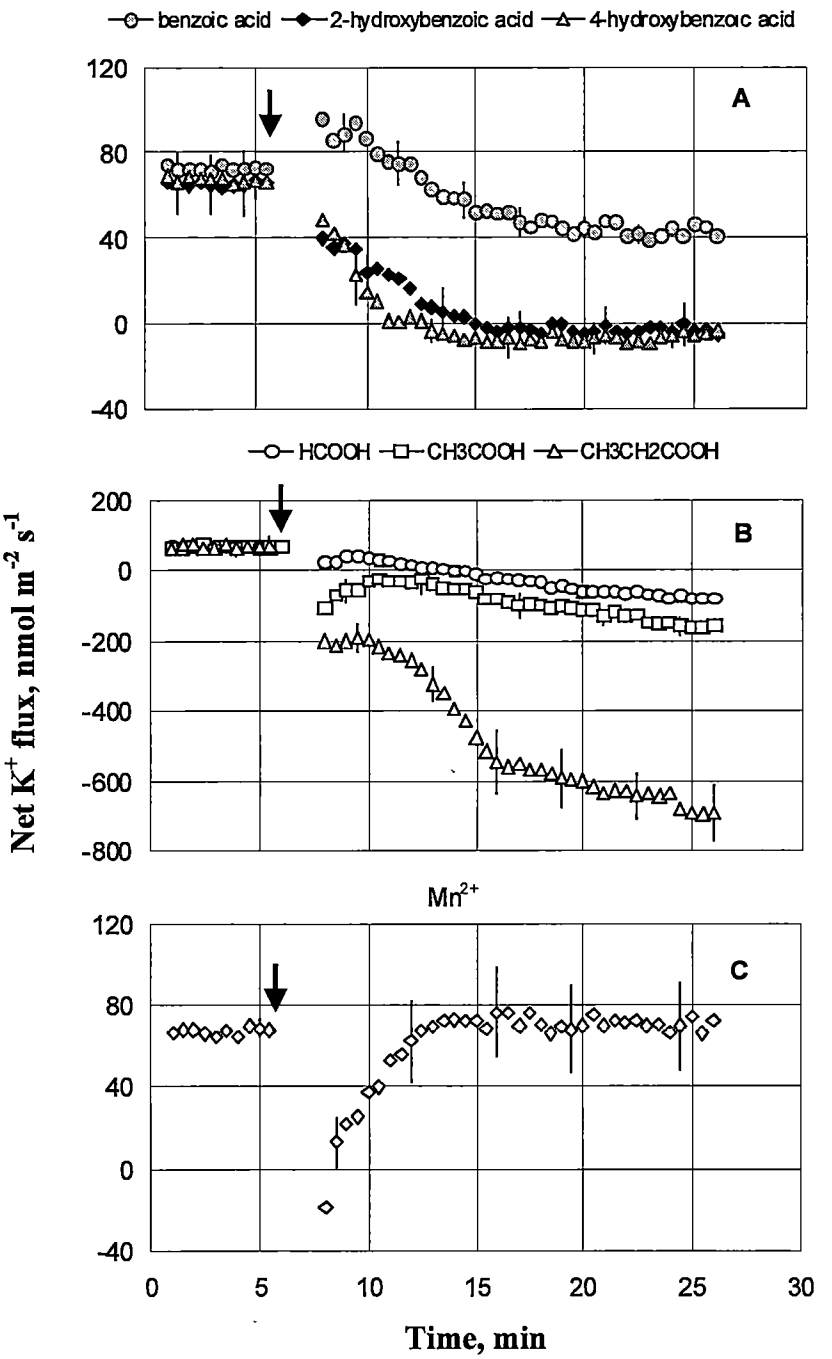

Fig. 6.1. K^+ flux kinetics in response to secondary metabolites associated with anaerobic soil conditions (applied at the time indicated by an arrow). (A) 200 µM phenolic acids treatment; (B) 10 mM monocarboxylic acids treatment; (C) 300 mg L^{-1} Mn^{2+} treatment. Measurements were made in the mature zone, 10-20 mm from the root tip. Data is mean ± SE (n = 8).

6.4.1.2. H^+ fluxes

Net H^+ efflux of 10 to 15 nmol m^{-2} s^{-1} was measured in control (steady state) conditions from barley roots. Application of all secondary metabolites significantly ($P < 0.01$) affected H^+ fluxes (Fig. 6.2). An immediate and significant shift towards net H^+ uptake was observed in response to all phenolic and monocarboxylic organic acids tested (Fig. 6.2A, B), while in the case of Mn^{2+} treatment net H^+ efflux was significantly ($P < 0.01$) reduced (Fig. 6.2C). Among phenolics, the effect followed the sequence: 4-hydroxybenzoic acid ≈ 2-hydroxybenzoic acid > benzoic acid. For monocarboxylic organic acids, responsiveness of H^+ flux followed the sequence: formic acid > acetic acid > propionic acid.

6.4.1.3. Ca^{2+} fluxes

Net zero Ca^{2+} flux was measured in control (steady state) conditions. Root treatment with phenolic acids led to a gradual and prolonged increase in net Ca^{2+} uptake (Fig. 6.3A). No significant ($P < 0.05$) difference between effects of various phenolic acids was found. Adding 10 mM monocarboxylic organic acids to the bath caused immediate and substantial Ca^{2+} efflux from barley roots (Fig. 6.3B). A similar trend was observed for Mn^{2+} treatment (Fig. 6.3C). In both cases, net Ca^{2+} flux recovered to its original (zero) value within 10 to 15 mins (Fig. 6.3B, C).

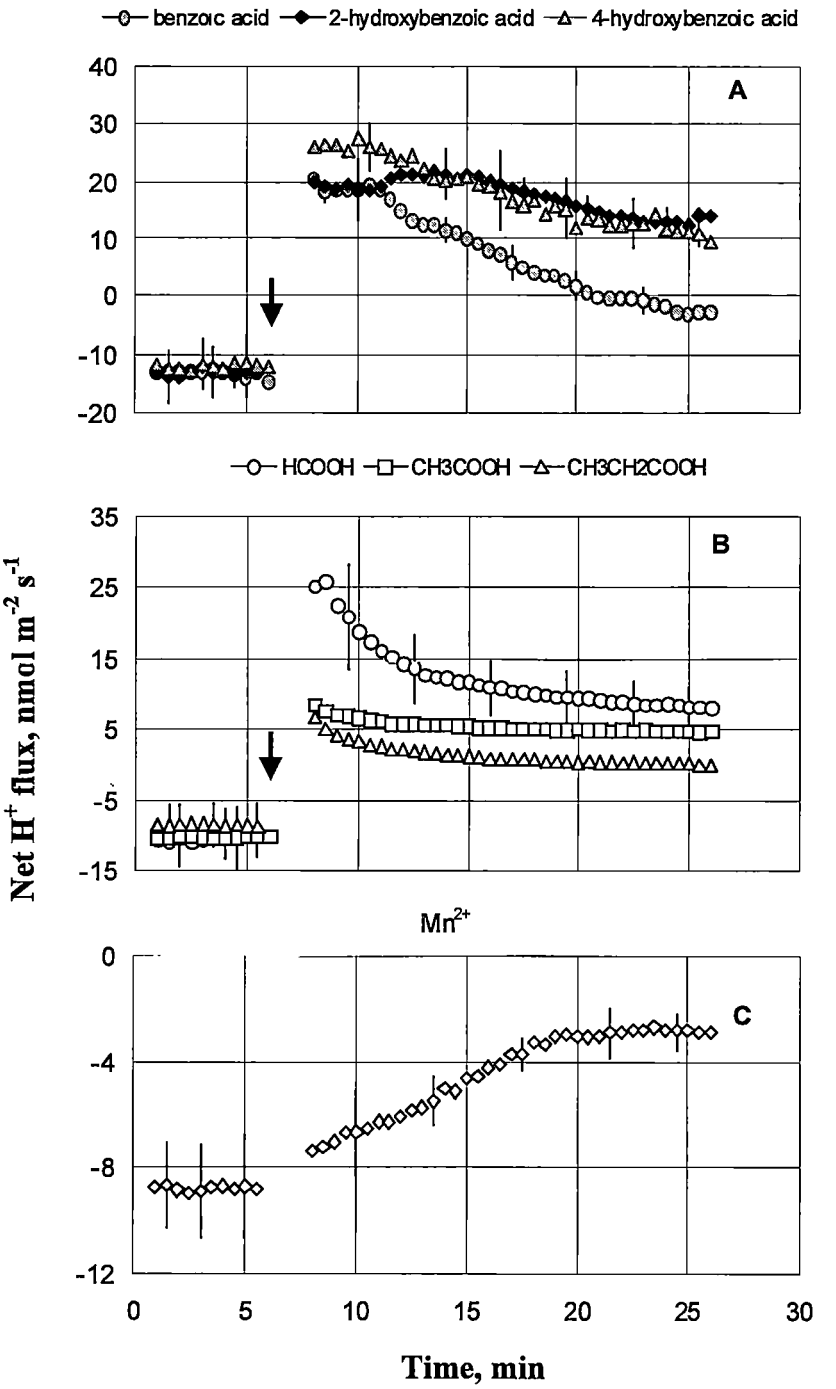

Fig. 6.2. H^+ flux kinetics in response to secondary metabolites associated with anaerobic soli conditions (applied at the time indicated by an arrow). (A) 200 µM phenolic acids treatment; (B) 10 mM monocarboxylic acids treatment; (C) 300 mg L^{-1} Mn^{2+} treatment. Measurements were made in the mature zone, 10-20 mm from the root tip. Data is mean ± SE (n = 8).

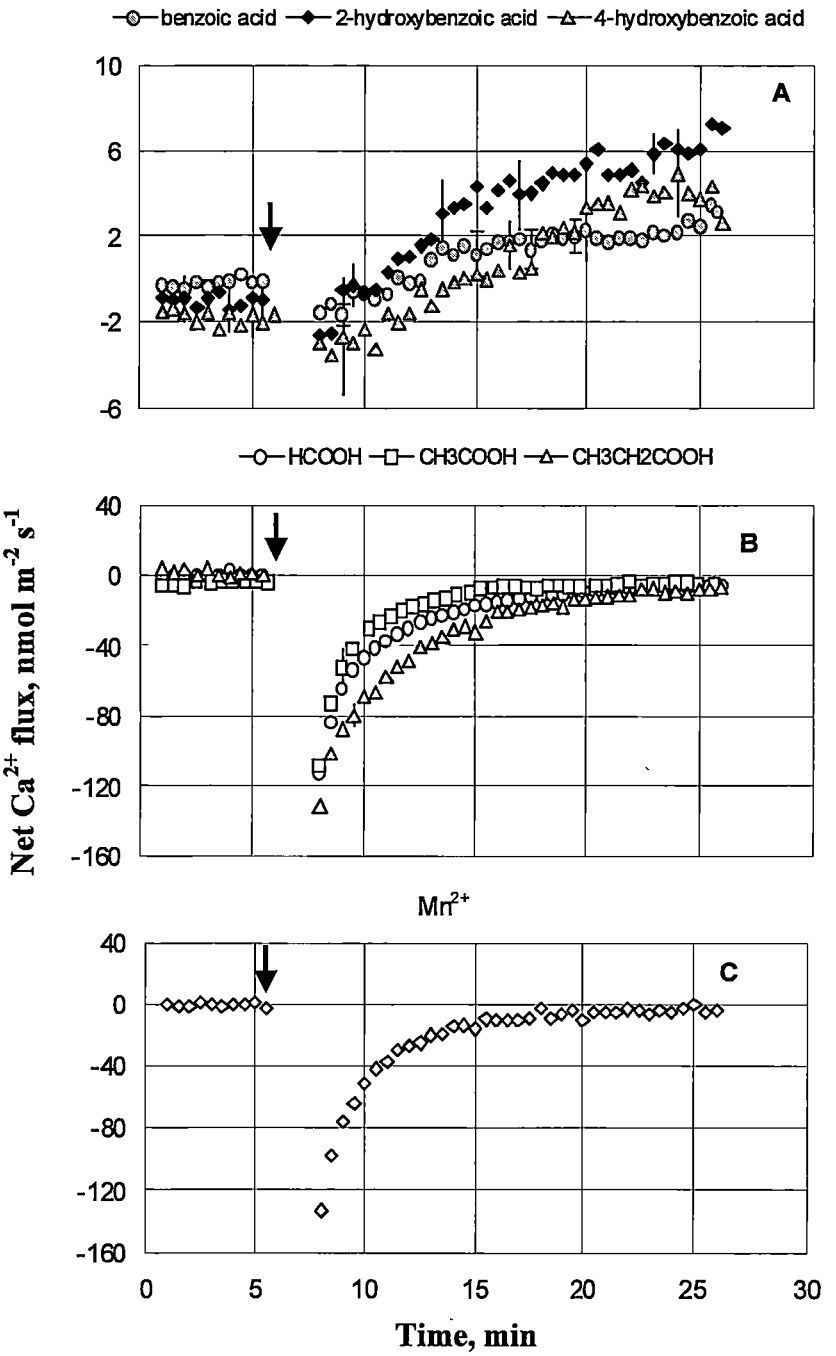

Fig. 6.3. Ca^{2+} flux kinetics in response to secondary metabolites associated with anaerobic soli conditions (applied at the time indicated by an arrow). (A) 200 µM phenolic acids treatment; (B) 10 mM monocarboxylic acids treatment; (C) 300 mg L^{-1} Mn^{2+} treatment. Measurements were made in the mature zone, 10-20 mm from the root tip. Data is mean ± SE (n = 8).

6.4.2 Long-term ion fluxes in response to secondary metabolites

6.4.2.1. K^+ fluxes

K^+ uptake was significantly reduced after 24 h treatment with all secondary metabolites tested (Fig. 6.4A). Root treatment with phenolic compounds (benzoic acid, 2-hydroxybenzoic acid, 4-hydroxybenzoic acid) caused significant ($P < 0.01$) decrease in net K^+ uptake. No significant ($P < 0.05$) difference between effects of various phenolic compounds was found. In monocarboxylic acid treated roots, K^+ fluxes were shifted to substantial (-40 to –100 nmol m^{-2} s^{-1}) net efflux. Among them, acetic acid and propionic acid caused more severe effects than formic acid. Mn^{2+} treatment also caused a net K^+ efflux. In general, the adverse effects of phenolic acids were smaller than the other 4 treatments.

6.4.2.2. H^+ fluxes

The monocarboxylic acid treatments reversed H^+ from net efflux to net influx (Fig. 6.4B). Mn^{2+} treatment reduced H^+ efflux to around zero. Among phenolic acids, 2-hydroxybenzoic acid and 4-hydroxybenzoic acid did not cause the significant ($P < 0.05$) change of H^+ fluxes, while benzoic acid slightly reduced H^+ efflux (Fig. 6.4B).

6.4.2.3. Ca^{2+} fluxes

Phenolic acids caused significant ($P < 0.05$) net Ca^{2+} efflux from roots pre-treated for 24 h (Fig. 6.4C). Formic and acetic acids also slightly reduced net Ca^{2+} uptake, while propionic acid and Mn^{2+} did not significantly ($P < 0.05$) affect Ca^{2+} fluxes (Fig. 6.4C).

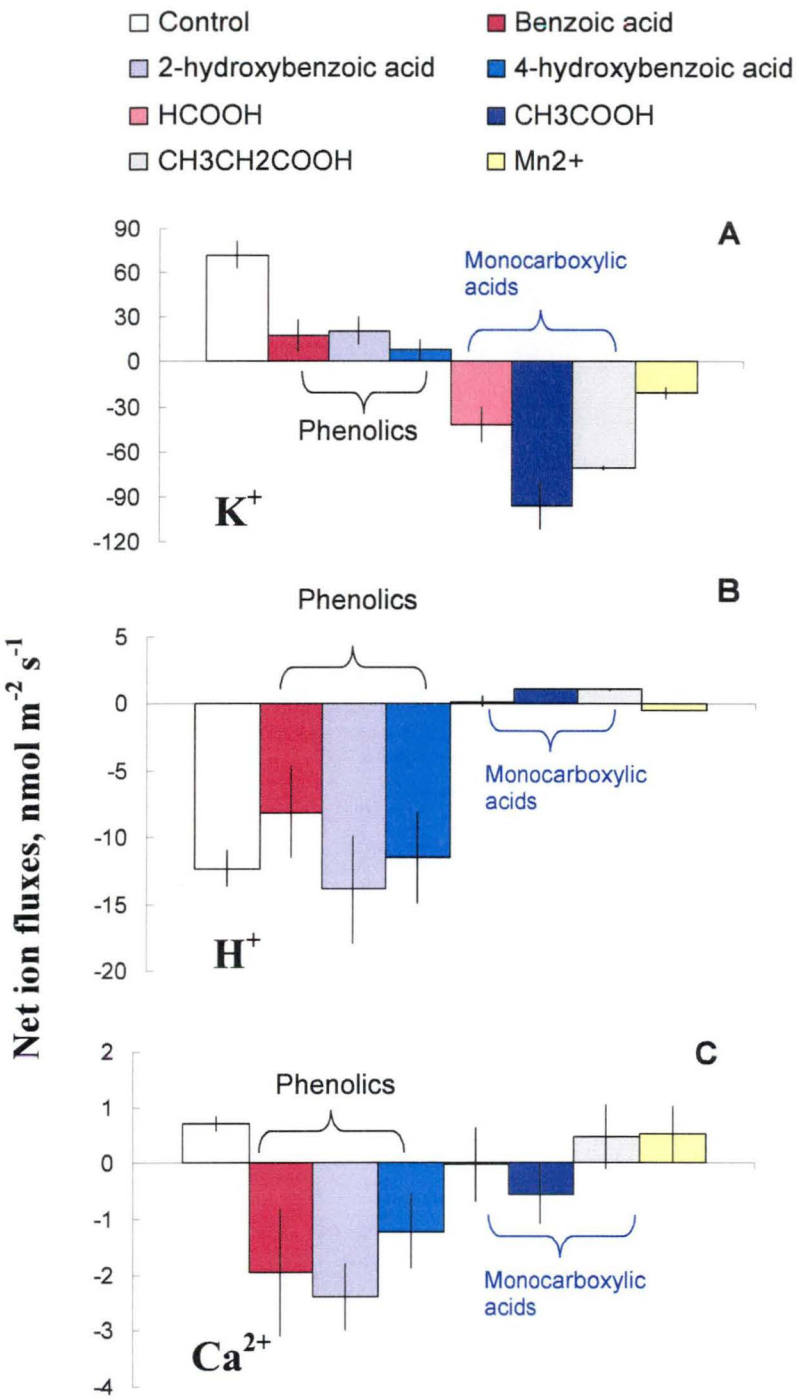

Fig. 6.4. Fluxes of K^+ (A), H^+ (B), Ca^{2+} (C) measured in the mature zone of barley root after exposed to various secondary metabolites for 24 hrs. Data is mean ± SE (n=12).

6.4.3 Membrane potential responses to secondary metabolites

6.4.3.1. Short-term response

The average membrane potential in the mature zone of barley roots was –133.9 ± 2.0 mV in control. Phenolic compounds caused substantial membrane depolarizaton (as illustrated in Fig. 6.5A for the treatment with 200 μM 2-hydroxybenzoic acid), stabilizing at –90 mV level approximately 10 min after the treatment was applied. Membrane potential kinetics in response to monocarboxylic compounds was not measured.

6.4.3.2. Long-term effects

Contrary to the short-term effects of 2-hydroxybenzoic acid, 24 h treatment with 200 μM phenolics caused significant ($P < 0.01$) hyperpolarization of membrane potential (Fig. 6.5B). The largest hyperpolarization effect was found in roots treated with 4-hydroxybenzoic acid. All 3 monocarboxylic acids and Mn^{2+} treatment induced significant ($P < 0.001$) depolarization of membrane potential (Fig. 6.5B).

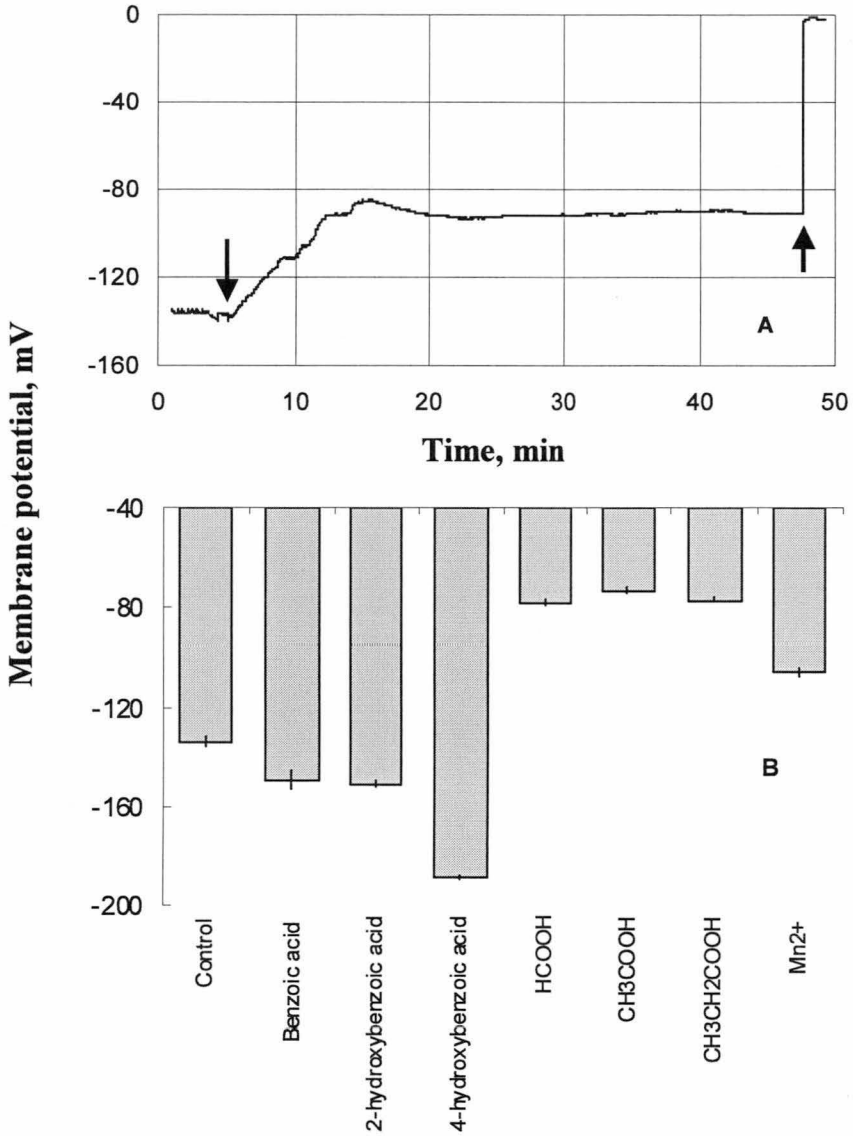

Fig. 6.5. A – A typical example of transient change of membrane potential upon the addition of 200 µM 2-hydroxybenzoic acid in root mature zones. The first arrow indicates commencing of the treatment, while the second arrow shows the moment when the electrode was removed from the cell. B – Cell membrane potentials in root mature zones after 24 h treatment with various secondary metabolites. Data is mean ± SE (n=16).

6.5. Discussion

6.5.1 Phenolics: short-term effects

In this study, root treatment with phenolic compounds (benzoic, 2-hydroxybenzoic, 4-hydroxybenzoic acids) resulted in a rapid shift towards net K^+ efflux from barley roots (Fig. 6.1A). This is generally in accord with early reports of Glass (1974b) who showed that all 13 benzoic compounds (500 μM) tested caused substantial inhibition of potassium absorption (measured as ^{86}Rb uptake from excised barley roots) after 3h of treatment. As ^{86}Rb measures a unidirectional K^+ uptake, however, it remained unclear to what extent K^+ efflux systems have been affected. My data (Fig. 6.1A) show that net K^+ fluxes from roots treated with 2-hydroxybenzoic and 4-hydroxybenzoic acids were close to zero or even slightly negative (net efflux), suggesting that both reduction in K^+ uptake and increase in K^+ leak took place. Also, much higher time resolution of the MIFE system (5 sec compared with several minutes in Glass' experiments) allowed a better quantification of the ion flux kinetics.

Among three phenolics, the effects of 2-hydroxybenzoic and 4-hydroxybenzoic acids on K^+ flux were larger than effects caused by benzoic acid (Fig. 6.1). Earlier Glass (1973) suggested that increasing hydroxylation within a series tends to decrease the inhibitory capacity of phenolics. This was obviously not the case in this experiments.

Under conditions of this experiment (pH 5.5), most of the phenolic acids in solution will be in dissociated form. The undissociated acid concentrations can be calculated according to Henderson-Hasselbalch equation:

$$pH = pK + \log_{10}([\text{conjugate base}]/[\text{conjugate acid}])$$

As shown in Table 6.1, the amounts of undissociated acids were relatively low and comprised 4.7%, 0.3% and 8.7% for benzoic, 2-hydroxybenzoic and 4-

hydroxybenzoic acids, respectively. Therefore, no obvious correlation between the magnitude of effect and the amount of dissociated compound was found.

Table 6.1. Concentrations of undissociated phenolic acids under conditions of experiment.

Compound	Dissociation constant, uM	pK	Concentration of undissociated compound, %
Benzoic acid	64.6	4.19	4.7
2-hydroxybenzoic acid	1071 (K_1)	2.97 (pK_1)	0.3
4-hydroxybenzoic acid	33.1 (K_1)	4.48 (pK_1)	8.7

The mechanisms by which phenolic compounds control K^+ transport across the plasma membrane remain elusive. Based on the fact that removal of phenolics caused a rapid recovery of K^+ reabsorption, Glass (1974b) suggested a direct effect on cell membranes. No specific details have been offered though. My data reported in this chapter suggests that major voltage dependent K^+ -transporting systems may be key players. This is supported by observed immediate membrane depolarization (Fig. 6.5A), which is also consistent with previous studies (Glass 1974a). Moreover, my data suggest that both increased H^+ (Fig. 6.2A) and Ca^{2+} (Fig. 6.3A) uptake could contribute to this depolarisation as depicted in Fig. 6.6A.

It is still unclear whether phenolic compounds can be transported across the cell membrane in a dissociated form. A hydrogen co-transport mechanism is shown to be the major avenue for uptake of major inorganic anions such as phosphate, sulphate, nitrate and chloride into the cell, as well as for most amino acids (Palmgren 2001, Palmgren 1998). If this were the case for phenolics (Fig. 6.6A), it could explain the observed increase in net H^+ uptake (Fig. 6.2A) measured in my experiments.

However, to the best of my knowledge, no experimental evidence supporting the above scenario is available in the literature. It was traditionally believed that most phenolic acids are crossing the cell membrane in an undissociated form by passive

diffusion (Jackson and St. John 1980). However, recent cloning and functional characterisation of the MCT1 family of transporters suggested that uptake of both monocarboxylic acids and benzoic acid occur via the H^+-coupled co-transport mechanism, at least in animal systems (Kido *et al.* 2000). As undissociated phenolic

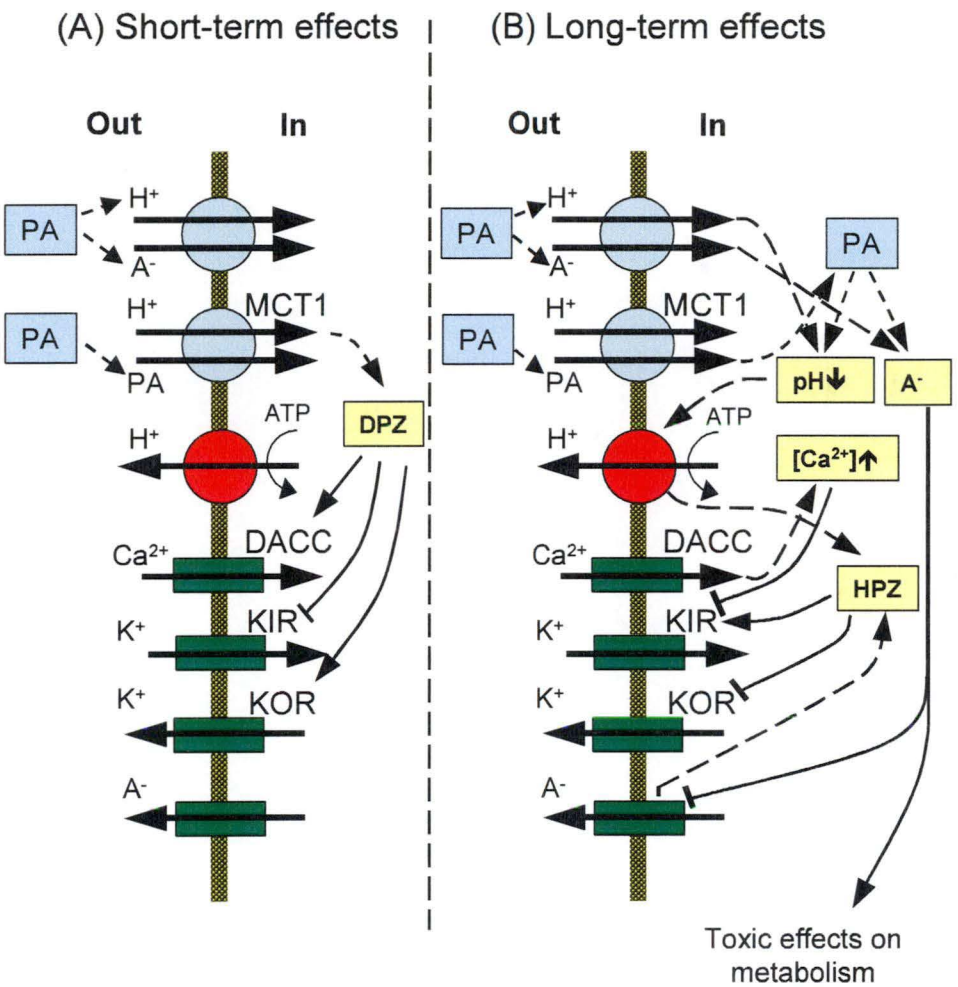

Fig. 6.6 A suggested short-term and long-term model explaining effects of phenolic acids on membrane transport activity. PA-phenolic acids; KIR- potassium inward-rectifying channel; KOR- potassium outward-rectifying channel; DACC – depolarization-activated Ca^{2+} channel; DPZ- depolarization of the plasma membrane; HPZ – hyperpolarization of the plasma membrane.

acid is electrically neutral, not only increased net H^+ flux will be generated as a result of such activity (consistent with my H^+ flux data; Fig. 6.2A), but also a substantial membrane depolarization is expected (Fig. 6.5). Such depolarization will affect intracellular K^+ homeostasis by reducing K^+ uptake via inward-rectifying K^+ channels (KIR) and enhance K^+ efflux via depolarization-activated outward-rectifying K^+ channels (KOR) (Fig. 6.6A). This will explain a rapid shift towards net K^+ efflux measured in my experiments (Fig. 6.1A).

Membrane depolarization can also lead to increased Ca^{2+} influx via depolarisation-activated Ca^{2+} channels (DACC) (Miedema *et al.* 2001, Thion *et al.* 1998). Depolarization-activated plasma membrane calcium channels have been suggested to play prominent roles in signal perception and transduction processes during growth and development of higher plants (Thion *et al.* 1998). Maximum Ca^{2+} current and Ca^{2+} influx is generally observed at about –100 mV, but this value may depend on the exact ionic composition of assay media (White 1998). Increased Ca^{2+} uptake will provide a positive feedback to further depolarize membrane potential, amplifying the effect of phenolics on K^+ transport (Fig. 6.6A).

6.5.2 Phenolics: long-term effects

Once inside the cell, permeated phenolic acids dissociate and acidify the cytosol (Ehness *et al.* 1997, Guern *et al.* 1986, Xiao *et al.* 2001). This will activate the plasma membrane H^+-ATPase and increase H^+ extrusion (Beffagna and Romani 1991, Felle 1989, Frachisse *et al.* 1988). As a result of such activation, net H^+ uptake observed in the first minutes after treatments with phenolics gradually decline, and membrane potential is expected to be restored. Indeed, after 24 h treatment, roots treated by each of the three phenolic acids had net H^+ flux values not significantly ($P<0.05$) different from the control (Fig. 6.4B), while membrane potential values were even more negative (hyperpolarized) compared with control roots (Fig. 6.5B). This is in a general agreement with previous studies reporting

that initial rapid acidification caused by weak acids was followed by a partial recovery of pH_{cyt} (Frachisse *et al.* 1988, Guern *et al.* 1986, Reid *et al.* 1989).

Theoretically, membrane hyperpolarization observed after long-term phenolic treatment (Fig. 6.5B) was expected to reverse detrimental effects of metabolites on K^+ transport. However, this was not the case, and K^+ uptake after 24 h of treatment with phenolic acids was significantly ($P<0.05$) lower than in the control (Fig. 6.4A). The answer may lay in the fact that net Ca^{2+} uptake measured soon after the treatment (Fig. 6.3A) may result in a substantial elevation in cytosolic free Ca^{2+} (Fig. 6.6B). Patch-clamp experiments on guard cells suggested that inward K^+ current is greatly reduced by elevating $[Ca]_{cyt}$ to micromolar concentrations (Schroeder and Hagiwara 1989). At the same time, outward-rectifying K^+ channels are much less sensitive to $[Ca]_{cyt}$ (Blatt and Grabov 1997, Grabov and Blatt 1997, Hosoi *et al.* 1988). Such differential sensitivity of KIR and KOR channels to elevation in cytosolic Ca^{2+} may shift the balance in net K^+ flux towards higher efflux and, thus, diminish beneficial effects of membrane hyperpolarization on K^+ nutrition.

It should be also mentioned that some benzoic acid derivatives (e.g. 5-nitro-2-(3-phenylpropylamino) benzoic acid) were found to be rather potent inhibitors of anion channels (Roberts 2006). At the same time, as a result of dissociation, a significant amount of organic anions will be accumulated in the cytosol. This accumulation might block their removal from the cytosol via anion channel (positive feedback), exacerbating toxicity effects. At the same time, anion channel blockage will add to observed membrane hyperpolarization, by reducing the amount of negatively charged particles leaving the cytosol. All these pathways are depicted in Fig. 6.6B.

6.5.3 Effects of monocarboxylic acids

Similar to phenolics, lipid-soluble undissociated forms of the volatile monocarboxylic acids are often regarded as most toxic (Jackson and St. John 1980, Jackson and Taylor 1970), and the amounts of undissociated monocarboxylic acids in my experiments could be calculated as the following (Table 6.2):

Table 6.2. Concentrations of undissociated monocarboxylic acids under conditions of experiment.

Compound	Dissociation constant, μM	pK	Concentration of undissociated compound, %
Formic acid	177	3.75	1.7
Acetic acid	17.6	4.75	15.1
Propionic acid	13.4	4.87	18.9

In this study, root treatment with all monocarboxylic (formic, acetic and propionic acids) compounds resulted in a significant ($P< 0.05$) net K^+ efflux from barley roots (Fig. 6.1). Of these, propionic acid caused the largest K^+ efflux, followed by acetic acid, and formic acid the least, proportional to the amount of undissociated acid in the bath solution (table 6.2). This is consistent with previous reports on the adverse effects of these acids on the K^+ uptake (Jackson and St. John 1980, Jackson and Taylor 1970).

Surprisingly, despite all my efforts, I failed to find any evidence for the direct effects of monocarboxylic acids on K^+ transport in plant, animal or bacterial systems. Early studies (Jackson and St. John 1980, Jackson and Taylor 1970) suggested that changes in membrane lipid composition might be responsible for the observed leak of K^+ and Ca^{2+} from roots treated with monocarboxolic acids. However, these conclusions were based on the overall measurements of ionic content in the root tissue, and had rather poor (e.g. hours) time resolution. Therefore, it is highly unlikely that such non-ion-specific change in general membrane permeability may occur almost immediately (within 1 min) after the

treatment, to explain the observed stimulation of K^+ efflux in my experiments (Fig. 6.1B). Such changes in a permeability are usually associated with the change in membrane lipid components (Glass 1974b, Jackson and St. John 1980, Jackson and Taylor 1970); the latter process is likely to operate at a slower time scale. Importantly, the above changes in membrane permeability are believed to be non-specific (Glass 1974a)) and, thus, a qualitatively similar kinetics for observed K^+ and Ca^{2+} leak should be observed. This was obviously not the case in this study. While K^+ leak gradually increased with time (Fig. 6.1B), Ca^{2+} efflux was short-lived and returned back to control values within 10-15 min after the treatment. This suggests that fluxes of these two ions are mediated by different transport systems and thus cannot be attributed to general change in membrane permeability.

A plausible alternative explanation may be offered. Similar to my model for phenolics (Fig. 6.6), monocarboxylic acids are transported into the cytosol, most likely in undissociated form (Kido *et al.* 2000). Being the H^+-coupled co-transport mechanism, such transport through MCT will cause significant H^+ influx measured in my experiments (Fig. 6.2B). This might depolarise the membrane and cause K^+ efflux through depolarization-activated K^+ channel (Fig. 6.2B). This remains to be tested in direct experiments by measuring membrane potential kinetics. Interestingly, contrary to effect of phenolics, monocarboxylic acids did not cause any substantial increase in Ca^{2+} uptake (Figs 6.3A and B, respectively), indicating a specificity of regulation of Ca^{2+} signalling by these secondary metabolites. Most likely, brief Ca^{2+} efflux measured in my experiments may originate from the Donnan exchange between K^+ and Ca^{2+} in the cell wall (Shabala and Newman 2000). This remains to be proven in direct pharmacological experiments.

The above scenario is further supported by the results of long-term experiments (Fig. 6.4). While very substantial K^+ leak was measured 24 h after the treatment with monocarboxylic acids (Fig. 6.4A), no significant ($P<0.05$) Ca^{2+} leak was found (Fig. 6.4C). Thus, it is highly unlikely that the general changes in membrane

permeability were involved as suggested by Jackson and co-authors (Jackson and St. John 1980, Jackson and Taylor 1970). Strong membrane depolarization (Fig. 6.5) also supports the idea of voltage-gated control of activity of K^+-permeable channels by monocarboxylic acids.

6.5.4 Effects of manganese

Another critical compound potentially mediating detrimental effects of waterlogging on root growth is manganese. Manganese is mainly absorbed as divalent Mn^{2+}. Although an essential micronutrient for plant functions, it can be toxic when present in excess (Mukhopadhyay and Sharma 1991). A possible mechanism for Mn^{2+} uptake across the plasma membrane may be ZIPs (zinc-regulated transporter/iron-regulated transporter (ZRT/IRT1)-related proteins (Hall and Williams 2003). Recent studies have also shown that so-called yellow stripe (YS) and yellow stripe-like (YSL) proteins are involved in metal-complex transport at the plasma membrane in a range of plants including maize, rice and Arabidopsis (Roberts *et al.* 2004). Many grass species use phytosiderophores (PSs) to chelate metals in the soil environment before accumulation into the roots, and the latter metal-PS complex is transported by ZmYS1 (Pittman 2005). It has been demonstrated that ZmYS1 is energized by H^+-cotransport (Schaaf *et al.* 2004). This is consistent with significant ($P< 0.05$) increase in H^+ uptake (Fig. 6.2C) observed in my experiments. However, contrary to the effects of phenolic and monocarboxylic compounds, the effect of Mn^{2+} on K^+ transport was only short-lived (Fig. 6.1C), suggesting an efficient charge balance. At the same time, as the amount of Mn^{2+} in the cytosol increases (24 h treatment), the membrane becomes significantly ($P<0.05$) depolarized (Fig. 6.5), and K^+ efflux is evident (Fig. 6.4A). No evidence for effects of Mn^{2+} on Ca^{2+} uptake and signaling was found, with large Ca^{2+} efflux observed immediately after Mn^{2+} application being most likely a result of exchange between K^+ and Ca^{2+} in the Donnan system. Once again, the latter should be confirmed in pharmacological experiments.

Chapter 7. Amelioration of detrimental effects of waterlogging by foliar nutrient sprays in barley

7.1. Abstract

Six barley cultivars were subjected to waterlogging for 2 weeks in the glasshouse during the summer in 2004-2005. The adverse effects of waterlogging were significantly alleviated by the foliar spray of nutrient solution in all cultivars including improved shoot and root growth, reduced leaf senescence, increased chlorophyll content, photosynthesis, PSII effectiveness, and production of more adventitious roots compared with waterlogged plants with no extra foliar nutrient. Application of foliar nutrients did not cause any changes in root ion uptake in the short term, indicating no involvement of electric signalling between the shoot and the root. Auxin was found accumulated at the shoot base in waterlogged plants, and the spray of foliar nutrients significantly increased this accumulation in waterlogged plants after 14 days of treatment. Foliar application of 1-NAA also promoted the production of adventitious roots. The highest concentration of auxin was observed between 1.2 and 1.6 cm from the shoot-root junction. Foliar nutrient application also improved nitrogen and potassium content in both shoot and root, but Ca content was hardly influenced. It is concluded that the improvement of waterlogged plant growth by foliar nutrient spray could be related to both the improved plant nutrition and increased auxin accumulation in the shoot base.

7.2. Introduction

It is generally known that uptake and distribution of essential nutrients are perturbed in plants subjected to waterlogging (Drew 1988). As a result, plants usually show marked depression of N, P, K, Ca and Mg in the foliage grown in either

waterlogged soil or hypoxic nutrient solutions (Leyshon and Sheard 1974, Trought and Drew 1980b, Trought and Drew 1980c). Such changes could be due to several factors. Under hypoxia conditions, the reserve metabolic energy of root cells is considerably reduced as anaerobic metabolism only maintains it at a very low level (Drew 1997). As this energy is required for active transport of some mineral nutrients such as phosphate, nitrate, and potassium against their electrochemical gradients, their uptake is significantly decreased (Ashraf and Rehman 1999). Furthermore, nutrient transport to the shoots including N, P and K would tend to be reduced by the observed decreases in the root/shoot ratio after exposure to hypoxia for several days (Barrett-Lennard *et al.* 1988, Trought and Drew 1980c).

As shoot organs can also take up mineral nutrients, a common method of fertilization is the application of nutrients by spraying a solution onto the leaves (foliar nutrition) (Peuke *et al.* 1998). Uptake of the nutrients into the leaves could occur either by penetration through the stomata, or by diffusion through the cuticle. It has been shown conclusively that nitrogen, phosphorus, and potassium in soluble forms are readily absorbed by aerial plant parts, often several times more efficiently than from supplementary soil treatments (Andersson 1993, Wittwer and Teubner 1959, Xie and Zhang 2004). It was found that potassium is one of the most readily absorbed of the mineral nutrients by the leaves of plants (Bukovac and Wittwer 1957). Howard *et al.* (1998) reported that foliar application of potassium increased the average cotton lint yield up to 13%. Foliar application of boron increased the stability of leaf membranes, chlorophyll, soluble sugars, soluble proteins, amino acid contents, leaf RWC and dry mass accumulation in waterlogged maize (Sayed 1998). Foliar sprays of nitrogen fertilizers caused appreciable yield improvement in waterlogged cotton (Hodgson 1982), rape (Zhou *et al.* 1997) and soybean (Sugimoto *et al.* 1989) plants.

Although application of fertilizers to waterlogged soil has been frequently observed to improve plant growth (Drew 1988), the excess water in waterlogged soil causes a

very substantial dilution of efficient nutrient concentrations. In addition, the ability of plant roots to take up and transport mineral nutrients are significantly impaired under waterlogging stress (Ashraf and Rehman 1999, Malik *et al.* 2001), making nutrient delivery to the shoot not efficient. From this point of view, direct application of foliar nutrient sprays to shoots of waterlogged plants may be a much more efficient way of improving plant nutritional status. Validation of this hypothesis was one of the aims of this study. Several specific questions have been addressed. Will foliar nutrient application change the nutrient status in both shoots and roots of waterlogged plants? Will the change of nutrient status in plants be related to the production of more adventitious roots (a well-known adaptive feature of waterlogged plants)? If yes, what are the mechanisms underlying these changes? Are changes in plant hormonal status involved? Ethylene is reported to influence adventitious root formation and has been found to occur at elevated levels in flooded plants (He *et al.* 1996a, Visser *et al.* 1996b). On the other hand, it also has been found that a gradual accumulation of auxins in flooded plants may also be a controlling factor in the formation of adventitious roots (Phillips 1964). Wample and Reid (1979) also concluded that flood-induced adventitious root formation was stimulated primarily by an accumulation of auxins in the hypocotyls. As hormones play important roles in regulation of both root and shoot growth, another specific aim of this work was to study whether foliar nutrient application can influence the change of hormone status and whether the latter is involved in the production of adventitious roots.

7.3. Materials and Methods

7.3.1 Plant material

Six barley cultivars (Naso Nijo, a cultivar of Japanese origin; Franklin and Gairdner, two Australian cultivars; ZP, TX9425 and DYSYH, cultivars of Chinese origin) were studied in this work.

7.3.2 Growth conditions

Growth conditions were similar to those described in Chapter 4. Experiments were carried out in a glasshouse during the summer-autumn in 2004/2005. Plants were grown in 2-L pots filled with dark grey Vertosol soil (4 seedlings in each pot). Average daily/nightly temperatures varied between 22°C/14°C during growth. Plants were grown under natural light during daytime, supplemented by 400W mercury lamps during morning and evening (PPFD 400 μmol/m^2.s; day/night 16/8 h).

7.3.3 Experimental protocol

Foliar nutritional experiments: Two pots of each cultivar, with four seeds per pot, were put in a black plastic tank (12 pots with 48 seedlings in each tank), with 24 tanks being set up as six replicates of 4 tanks. Once the seedlings were 2 weeks old, three tanks per replicate were waterlogged, with the water level kept at the soil surface. One of these tanks was sprayed daily with ¼ strength Hoagland nutrient solution (labelled as **1/4FHWL** in following graphs), and another sprayed with full strength Hoagland solution (**FHWL**), 10ml for each plant. The full-strength Hoagland nutrient solutions contained (in mol m^{-3}): $MgSO_4$, 2.0; $Ca(NO_3)_2$, 5.0; KNO_3, 5.0; $NH_4H_2PO_4$, 1.0, together with micronutrients and iron-EDTA (Hoagland and Arnon 1938). The treatment was maintained for 2 weeks. The other waterlogged tank in each replicate received no nutrients, and the fourth tank was the non-waterlogged control also receiving no foliar nutrients. The experiment was carried out as a randomized split plot design with each tank as a main plot, and the cultivars as subplots.

Hormonal experiments: Two cultivars with contrasting waterlogging tolerance (Naso Nijo and TX9425) were used. A two-factor completely randomized block design (cultivar, treatment) was used with 5 replicates. Treatments included: control (well-drained), waterlogging (WL), waterlogging + full strength Hoagland solution

(FHWL), control + foliar NAA, waterlogging + foliar NAA, control 2 (well-drained + foliar ethanol + 0.1% Tween 20; the latter chemicals were used to dissolve synthetic auxin, 1-NAA – see below). Treatments were imposed for 2 weeks, starting when plants were 2 weeks old. Plants from control, WL and FHWL were sampled twice, the first sampling being taken after 5 days of treatment and the second after 14 days of treatment. The foliar application of 1-Naphthaleneacetic acid (1-NAA) was done as described by McDonald and Visser (2003). 1-NAA was first dissolved in a small volume of ethanol (final ethanol concentration 0.5%) and then made into an aqueous solution with the addition of 0.1% Tween 20. 5 μg 1-NAA (in 200 μL of solution) was applied to the shoot of each plant in control and waterlogging plants with a small brush. Control 2 plants were brushed with 200 μL of deionized water with 0.5% ethanol + 0.1% Tween 20.

Electrophysiological experiments: For the MIFE measurement, Gairdner plants were grown hydroponically in 2 L containers with the aerated nutrient solution described in experiment 1 in laboratory at 21°C until the appearance of 2 fully expanded leaves.

7.3.4 Basic plant characteristics

Two weeks after the beginning of treatment, a sample of 12 each of the control and stressed plants was harvested and all soil was carefully washed off plants. The longest root was measured with a rule. The number of adventitious roots was counted manually. Plant material was dried at 65°C in a Unitherm Drier (Birmingham, England) and then the shoot and root dry weights were determined. The number of chlorotic leaves was visually scored.

7.3.5 Chlorophyll fluorescence

Chlorophyll fluorescence was measured at a temperature of 20°C ± 3°C with a pulse-amplitude modulation portable fluorometer (Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany). Details are described in Section 3.4.4.

7.3.6 Pigment analysis

Extractions of chlorophyll *a* and *b* were made 2 weeks after waterlogging. A fresh weight sample of ~0.1g was taken, from the youngest fully expanded leaf. Eight replicates for each of the cultivars and treatments were analysed and chlorophyll content was averaged over the eight replicates. Details are described in Section 3.4.3.

7.3.7 CO_2 assimilation

Gas exchange was measured with a LCI Portable Photosynthesis System Infrared Gas Analyzer (ADC BioScientific Ltd, Hoddesdon, England) on the youngest fully expanded leaves after 2 weeks of treatment in the foliar nutritional experiment. Details are described in Section 3.4.5.

7.3.8 Stomata conductance

Stomatal conductance was measured using a Delta-T MK3 porometer (Delta-T devices, Cambridge, UK). Measurements were taken on a sunny day from the youngest fully expanded leaves.

7.3.9 Auxin content quantification

Shoot bases of 2cm long from the root-shoot junction after 5 and 14 days of waterlogging, with 5 replicates per treatment were sampled and put in pre-cooled 80% methanol + butylated hydroxytoluene (BHT, 1g L^{-1} methanol) immediately. For the extraction of auxin, tissue was homogenized and the extracts were held at 4 °C for 24 h before filtering (Whatman No. 1). IAA was quantified using [$^{13}C_6$] IAA as an internal standard. Sep-Pak C18 cartridges were used to purify all samples. Auxin samples were derivatized by first adding 10 µL of pyridine and 40 µL of bis-trimethylsilyltrifluoroacetamide with 1% (v/v) trimethylchlorosilane, followed by heating at 80 °C for 20 min. Samples were then dried, and a further 15 µL of bis-

trimethylsilyltrifluoroacetamide with 1% (v/v) trimethylchlorosilane was added, followed by heating at 80 °C for 15 min. Then the samples were dried under N_2, taken up in about 25 µL of chloroform and transfered into an autosampler vial. Gas Chromatography and Mass Spectrometry (GC-MS) were performed as described by Ross (1998). The GC-MS system consisted of a Hewlett-Packard 5890 GC coupled to a Kratos Concept ISQ mass spectrometer. A Hewlett-Packard 25m* 0.32mm inner diameter * 0.17µm film HP1 column was used. The oven temperature was programmed from 60°C to 150°C at 30°C min^{-1} and then at 10°C min^{-1}, with a column head pressure of 15 p.s.i.

7.3.10 Tissue N, K and Ca content

Dry plant material was ground and 0.3-1.0g taken and placed in a digestion tube. 2.5 ml of the digestion mixture (concentrated H_2SO_4, Se and salicylic acid) was added. After mixing, the tube was allowed to stand for 2h and was then placed into a heating block for 2 h at 100 °C. After cooling, three lots of 1 ml H_2O_2 were added. After each addition, the contents of the tube were thoroughly mixed. Then the tube was placed into the heating block again and heated to 370 °C until the solution in the tube was clear. The cooled digest was diluted to 50 ml with distilled water, filtered and aliquots were taken for nutrient analysis. Nitrogen content was analysed using a Kjeltec system 1002 distillating unit. Potassium content in plant tissues was measured using an EEL flame photometer (Evans Electroselenium Ltd., Halstead, England). Ca content was analysed using an atomic absorption spectrometer (Varian, Melbourne, Australia).

7.3.11 MIFE experiments

To investigate whether foliar applied nutrients can be taken up by roots under control of some electrical signal propagating from the shoot to the root, an experiment was devised to measure ion exchange at the roots when leaves were subjected to treatment. Net fluxes of H^+, K^+ and Ca^{2+} were measured using the non-

invasive MIFE® system (University of Tasmania, Hobart, Australia). Details were described in Section 3.5 and chapter 5.

As the presence of cuticles in leaves might block the immediate uptake of nutrients, the cuticle in the youngest fully expanded leaves was removed by the gentle rubbing of the abaxial leaf surface with a cotton bud wetted with 15% ethanol for 5 s followed by thorough rinsing in running distilled water for several minutes as described elsewhere (Zivanovic *et al.* 2005). This caused no detrimental effects on leaf physiology, as judged by both electrophysiological characteristics and growth data (Zivanovic *et al.* 2005). Then the leaf was immersed into the same nutrient solution for the measurement of ion fluxes around the roots.

7.3.12 Statistical Analysis

Data for all growth and physiological parameters were subjected to analysis of variance (ANOVA) with General Linear Model (GLM) using the Minitab Statistical Program (Minitab Release 13.2, Minitab Inc., USA). Differences among treatments were compared using the LSD at the 0.05 level of probability.

7.4. Results

7.4.1 Photosynthetic characteristics

Two weeks of waterlogging caused a significant ($P<0.05$) reduction of leaf CO_2 assimilation (Fig. 7.1A). Foliar spray with ¼ strength Hoagland solution (1/4FHWL) did not significantly ($P<0.05$) improve leaf photosynthetic rate in any cultivar, while net CO_2 assimilation was significantly ($P<0.05$) increased in waterlogged plants sprayed with full strength Hoagland solution (FHWL) in most studied cultivars except in Franklin and DYSYH ($P=0.05$) (Fig. 7.1A). As a result, the shoot biomass was improved significantly in FHWL treated plants compared with WL plants in all cultivars ($P=0.05$) (Fig. 7.1B).

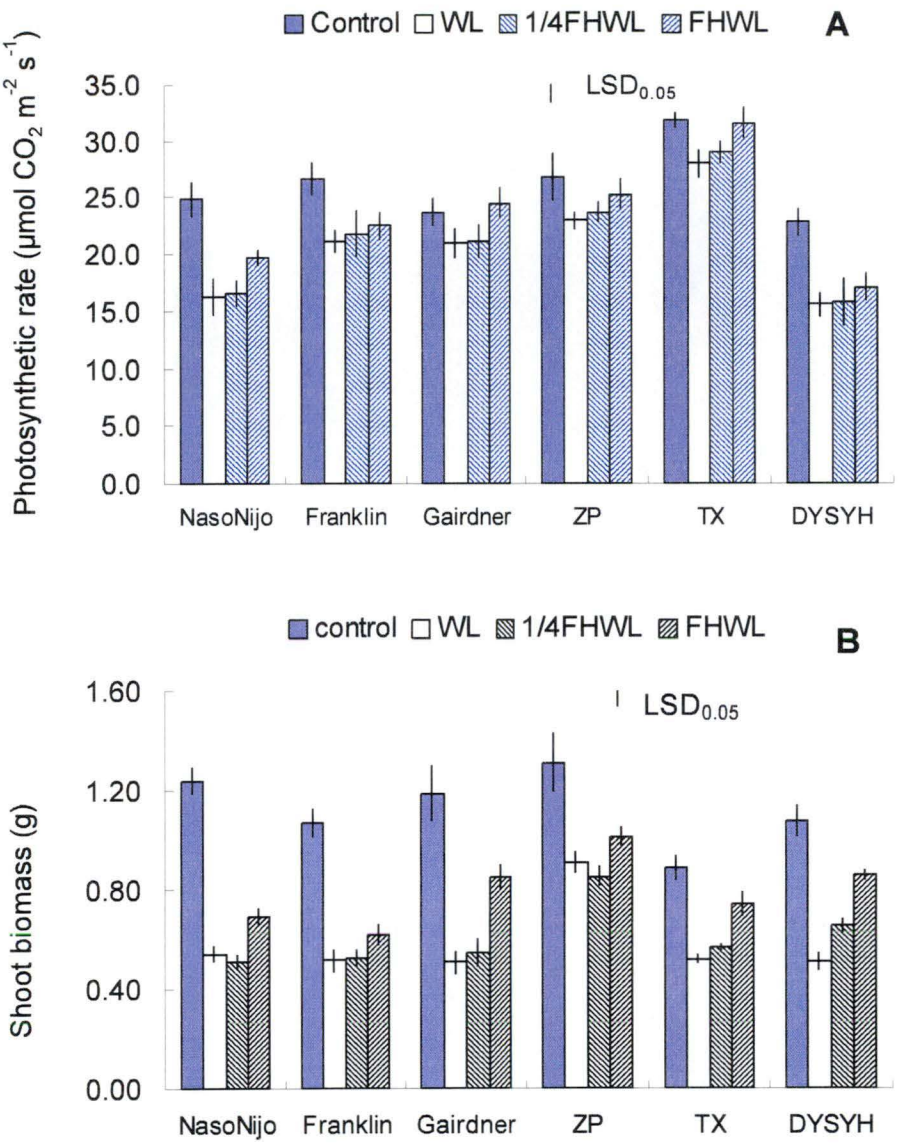

Fig. 7.1. Changes in photosynthetic rate (A) and the shoot biomass (B) after 2 weeks treatment in 6 barley cultivars. Error bars are SE (n=6 and 12 for A and B, respectively).

Waterlogging for 2 weeks reduced the chlorophyll content in all cultivars (Fig 7.2A). Both 1/4FHWL and FHWL treatments ameliorated adverse effects of waterlogging on leaf pigment composition in all cultivars. Very significant ($P<0.05$)

alleviation effects were found in Gairdner, ZP, TX and DYSYH, however, there was significantly less chlorophyll in FHWL plants compared with the control plants ($P<0.05$) (Fig. 7.2A).

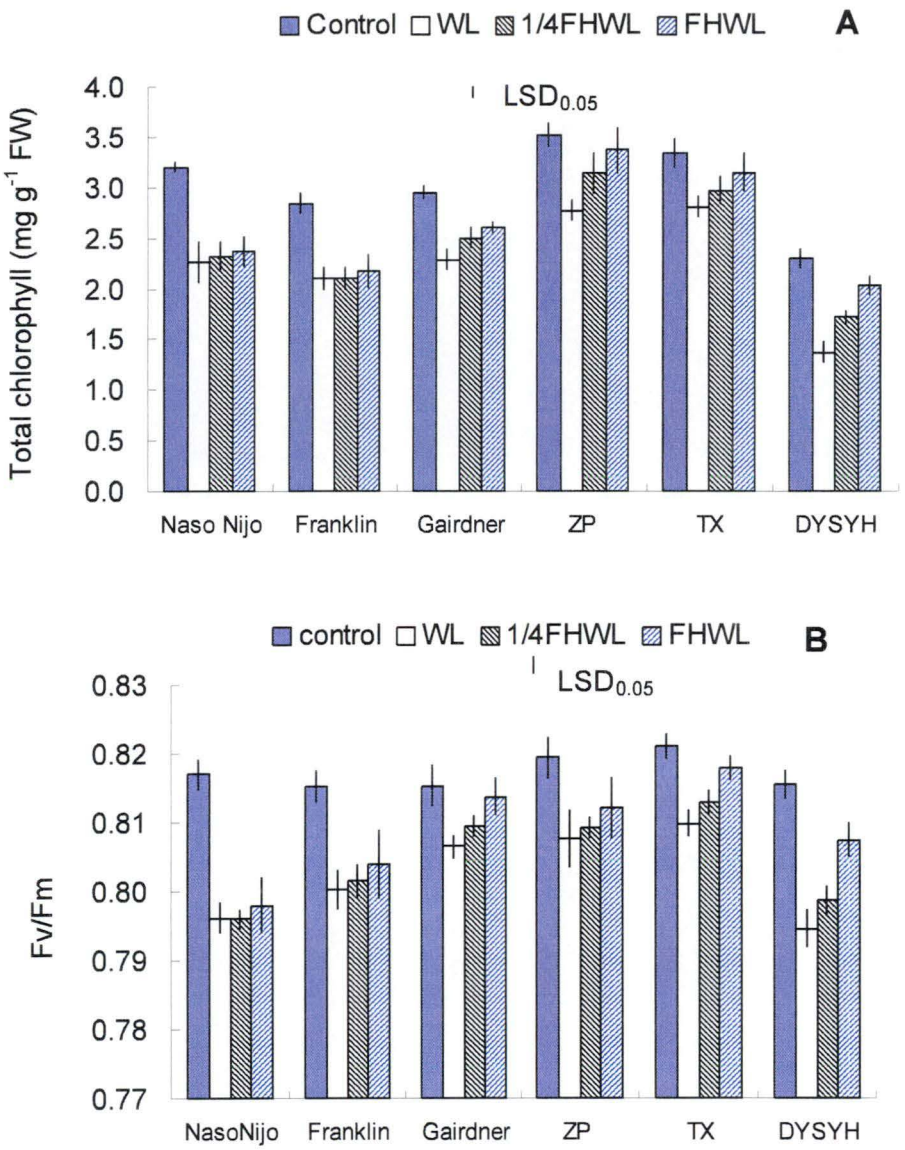

Fig. 7.2. Change in chlorophyll content (A) and chlorophyll fluorescence (B) after 2 weeks treatment in 6 barley cultivars. Error bars are SE (n=10)

After 2 weeks of treatment, Fv/Fm in waterlogged plants was significantly reduced in all cultivars compared with control plants ($P<0.05$); decrease in TX, ZP and Gairdner was smaller than in the other three cultivars. Compared with the waterlogged plants, ¼FHWL treatment showed no significant alleviation in Fv/Fm for most cultivars ($P<0.05$), while it was significantly improved for FHWL treatment in all cultivars except Naso Nijo. For TX and Gairdner, Fv/Fm in FHWL plants was as high as in control plants (not significantly different at $P<0.05$) (Fig. 7.2B).

7.4.2 Shoot nutrient content

WL caused significant ($P< 0.05$) reduction of nitrogen in shoots in all 4 cultivars (2 WL tolerant and 2 WL sensitive cultivars) compared to the control (Fig. 7.3). Potassium content was also significantly ($P<0.05$) reduced in all cultivars except TX compared with the control (Fig. 7.3). FHWL treatment alleviated the reduction of both N and K in the shoots of WL plants. WL had less effect on Ca in TX and ZP than in Naso Nijo and Franklin. However, FHWL did not alleviate the substantial reduction of Ca caused by WL in barley shoots in any cultivar (no significant effects at $P<0.05$).

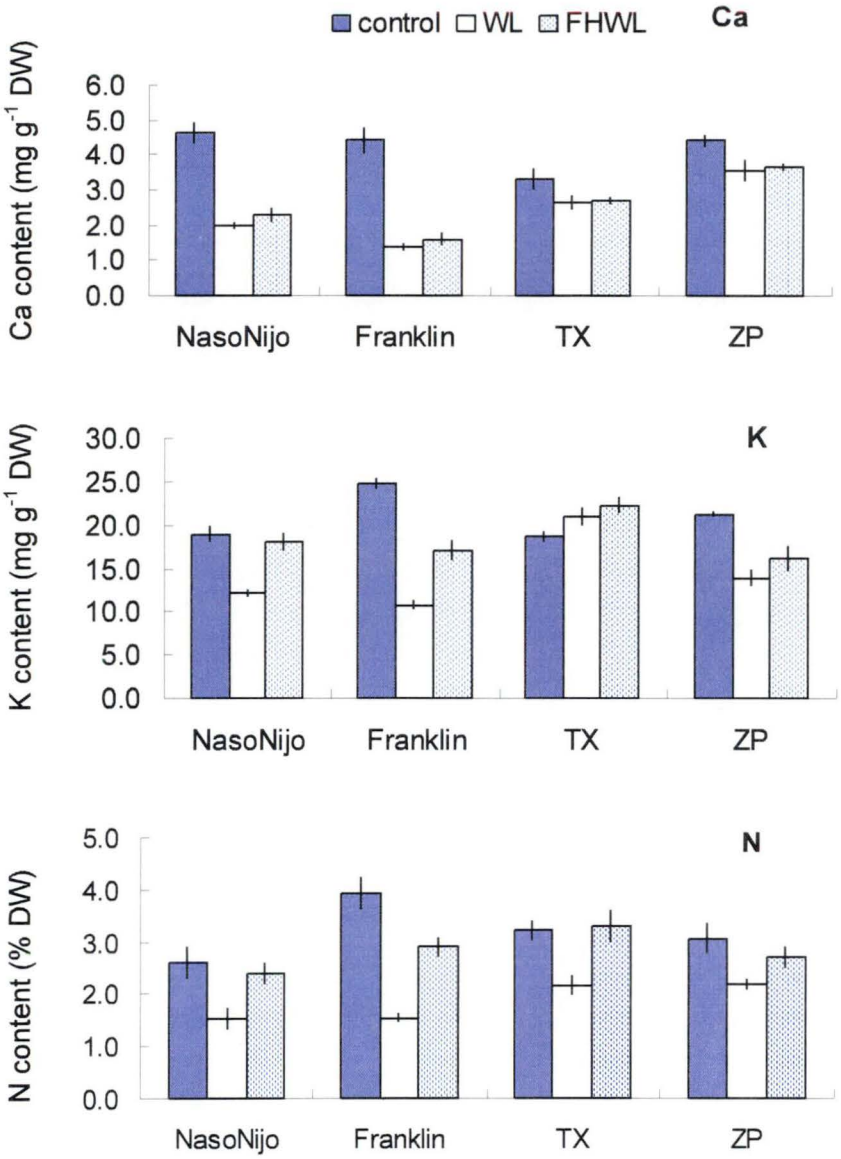

Fig. 7.3. Nutrient (Ca, K, N) content in shoots in 4 barley cultivars. Error bars are SE (n=12)

After 2 weeks of waterlogging treatment, the percentage of chlorotic leaves (a visual symptom of nitrogen deficiency) significantly ($P< 0.05$) increased compared with the control plants, in which no chlorotic leaves were observed in any of the barley cultivars studied (Fig 7.4). Among these six cultivars, TX9425 and DYSYH

showed the lowest chlorotic leaf percentage, followed by ZP, while Naso Nijo, Franklin and Gairdner showed much higher values under waterlogging conditions (Fig. 7.4). In 1/4FHWL or FHWL treated plants, the percentage of chlorotic leaves still showed a significant increase compared to the control plants. Leaf chlorosis was significantly ($P<0.05$) reduced for all FHWL plants while no significant difference was found for 1/4FHWL plants in all cultivars except in Franklin (Fig. 7.4).

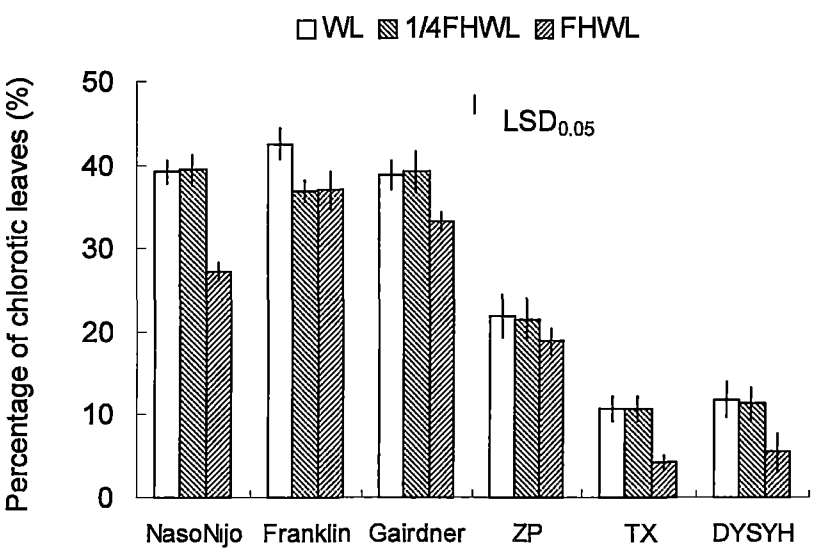

Fig. 7.4. Percentage of leaf chlorosis after 2 weeks treatment in 6 barley cultivars. Error bars are SE (n=12)

7.4.3 Root growth characteristics

Two weeks of waterlogging caused a significant ($P<0.05$) reduction of root biomass in all 6 cultivars (Fig. 7.5A). The root growth was significantly ($P<0.05$) improved by 1/4FHWL and FHWL treatments in all cultivars except for the effects of 1/4FHWL in Naso Nijo. FHWL was more efficient compared with 1/4FHWL in ameliorating detrimental effects of waterlogging on root biomass (Fig. 7.5A).

The length of the longest root in WL, 1/4FHWL and FHWL plants was significantly ($P<0.05$) reduced compared with the control (Fig. 7.5B). Waterlogged plants

produced mainly adventitious roots, as most seminal roots died after 2 weeks stress (data not shown). Among WL, 1/4FHWL and FHWL plants, no significant difference in the longest root length was found (P=0.05) (Fig. 7.5B). This suggests that the improvement in root dry weight in FHWL treatment was not attributed to the overall increase in the root length, but due to the production of more adventitious roots.

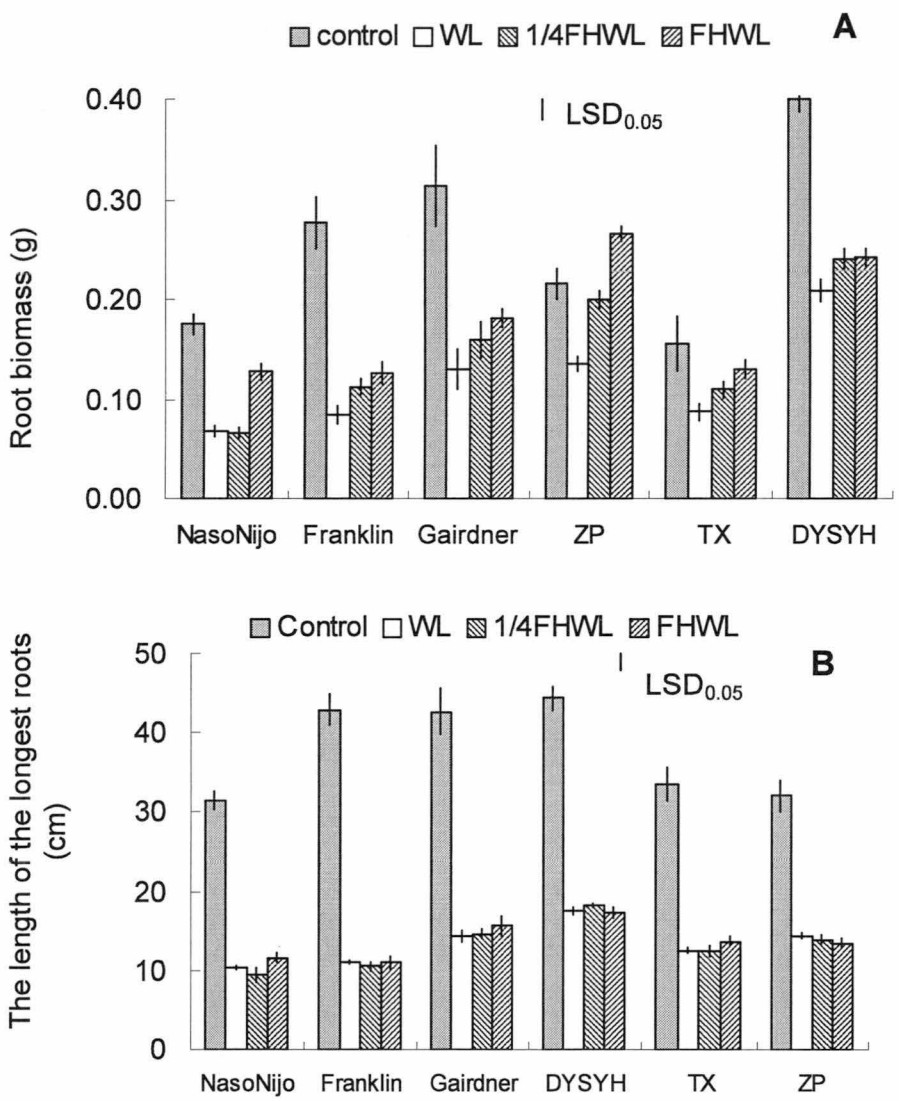

Fig. 7.5. Root biomass (A) and the length of the longest roots (B) in 6 barley cultivars after 2 weeks treatment. Error bars are SE (n=12).

7.4.4 Root nutrient acquisition

In order to investigate of whether application of foliar sprays can trigger electrical signalling and thus alter the rate of nutrient uptake in plant roots, a series of electrophysiological experiments was performed. Immersing one intact expanded leaf into the nutrient solution did not caused any change in the fluxes of K^+, H^+ and Ca^{2+} in barley roots in control plants (non-waterlogged) within 50 min of the treatment (Fig. 7.6).

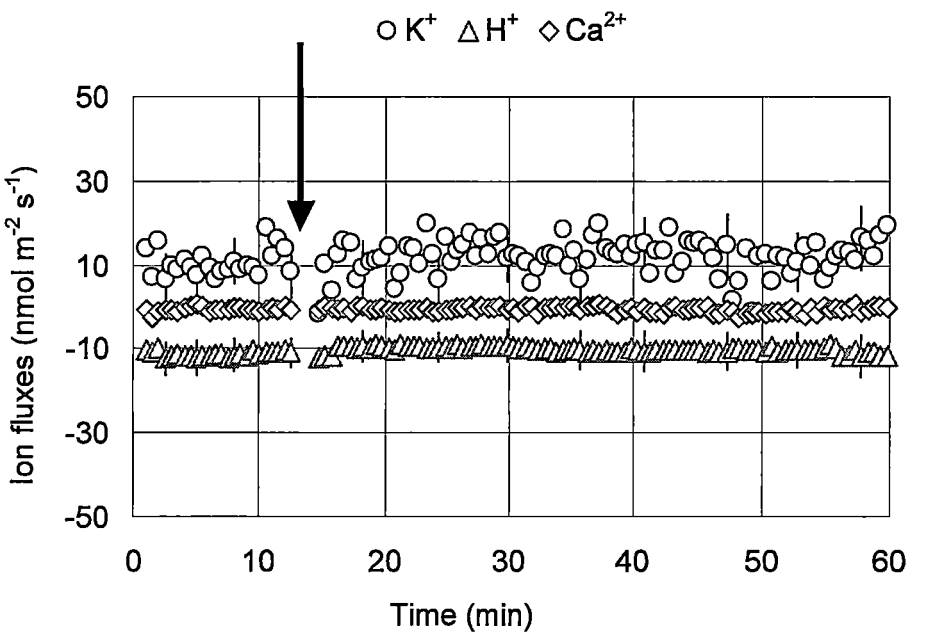

Fig. 7.6. K^+, H^+, Ca^{2+} ion fluxes in root mature zone upon the immersion (indicated by an arrow) of one mature leaf into the nutrient solution. Error bars are SE (n=8).

Waterlogging (WL) for two weeks resulted in reduced N in roots of all cultivars, and reduced K in all but TX (Fig 7.7). Two weeks of FHWL treatment significantly ($P<0.05$) increased K and N content in roots in cultivars studied compared to those in WL (Fig. 7.7). At the same time, there were no significant differences in Ca in roots of waterlogged or control plants (Fig. 7.7).

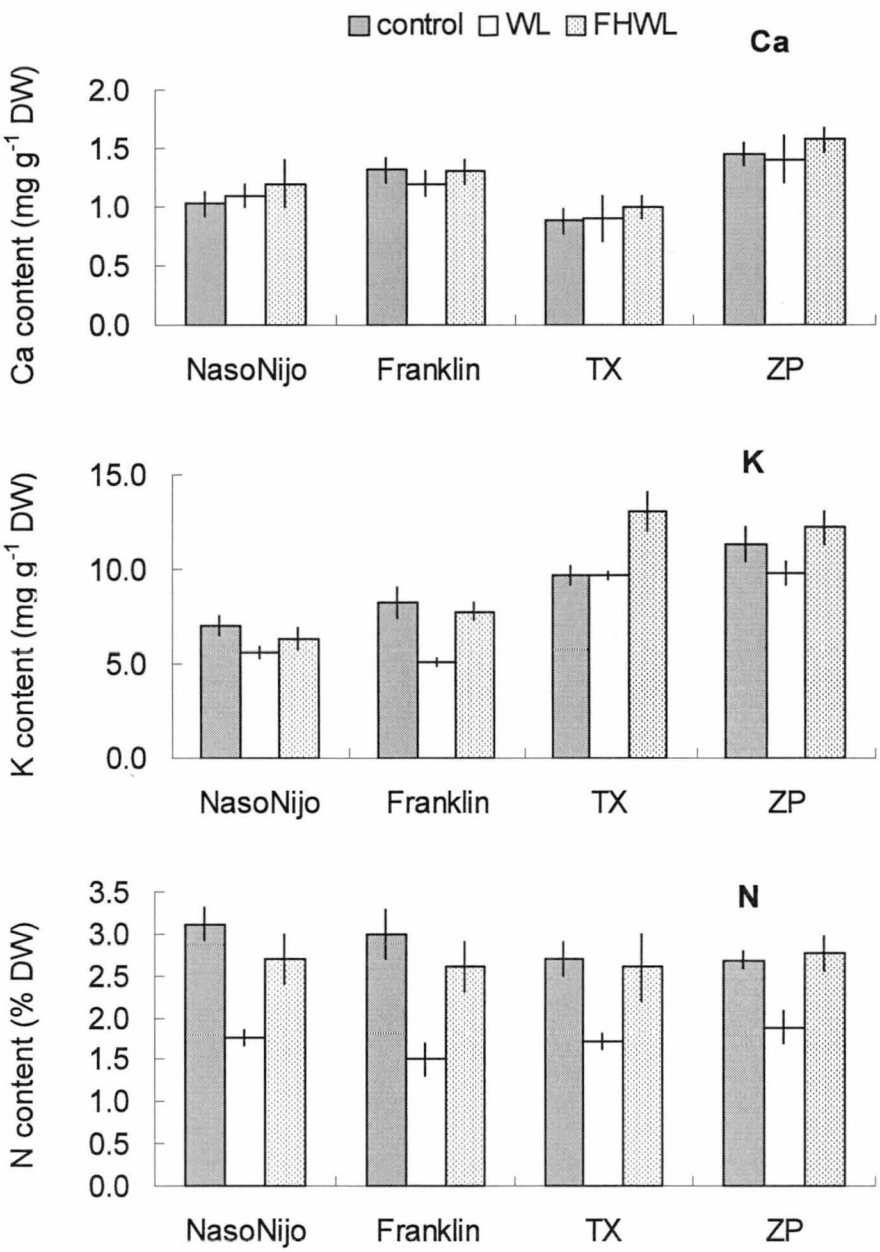

Fig. 7.7. Nutrient (Ca, K, N) content in roots of 4 barley cultivars after 2 weeks treatment. Error bars are SE (n=12)

7.4.5 Kinetics of ameliorative effects of foliar sprays

Five days after waterlogging, both root and total fresh weight were reduced significantly ($P<0.05$) compared with the control. At the same time, no significant difference between WL and FHWL treatments were found in either sensitive (Naso

Nijo) or tolerant (TX9425) cultivars (Fig. 7.8A and B), indicating the absence of ameliorative effects of foliar sprays in short terms.

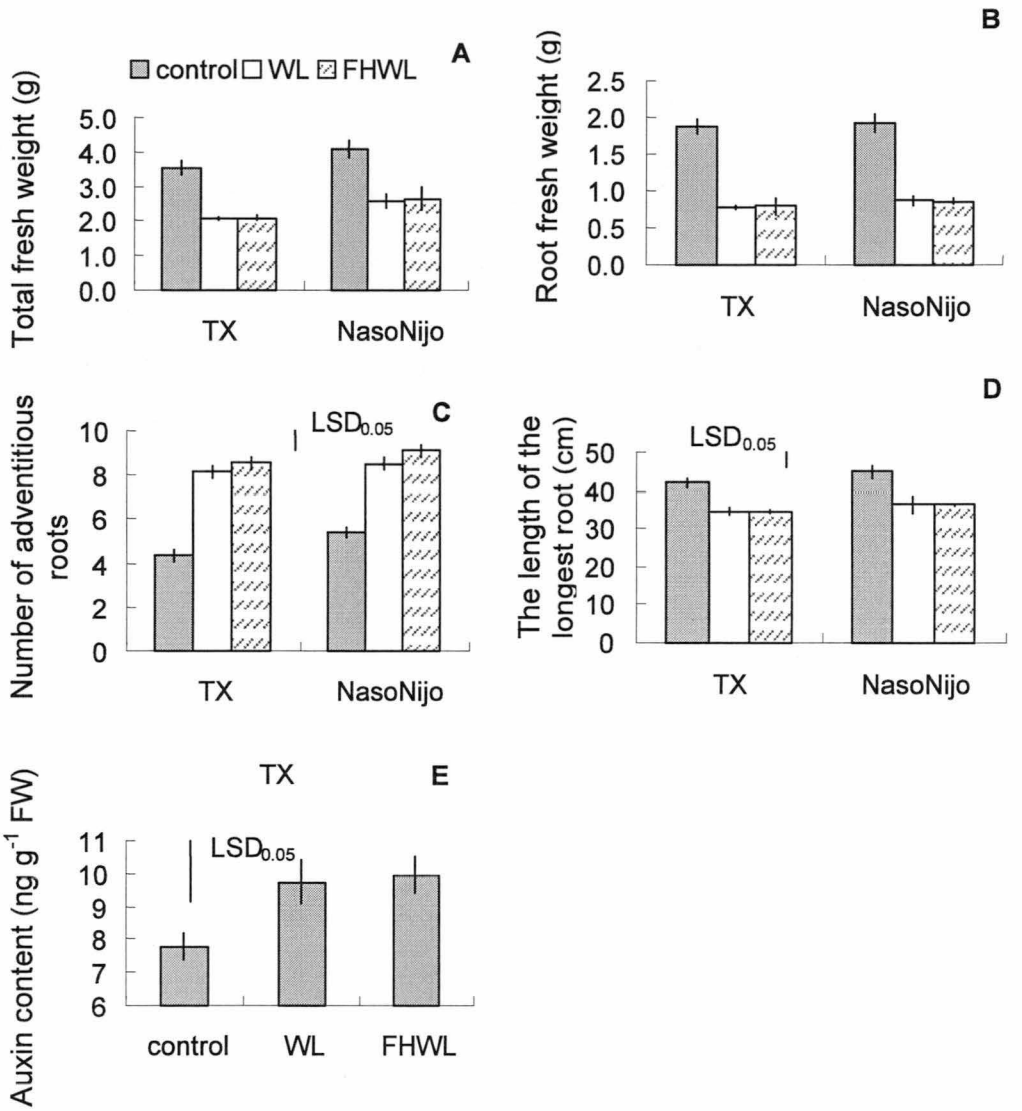

Fig. 7.8. Effects of 5 days of waterlogging on total fresh weight (A), root fresh weight (B), the number of adventitious roots (C), the length of the longest root (D) in TX9425 and Naso Nijo, and auxin content in TX9425 (E). Error bars are SE (n = 12 in A, B, C; n = 20 in D; n = 5 in E).

Both WL and FHWL treatments led to a substantial production of adventitious roots and the reduction of the length of the longest roots compared with the control (both

significant at $P< 0.05$). However, there was no significant difference between WL and FHWL treated plants after this short time (Fig. 7.8C and D). Five days of waterlogging also caused an increase in auxin content in the shoot base (within 2cm to the shoot-root junction). Again, no significant ($P < 0.05$) difference in auxin content between WL and FHWL treatments was found (Fig. 7.8E).

After two weeks of waterlogging, a significant increase in number of adventitious roots was found in WL and FHWL treatments compared with control plants in both Naso Nijo and TX cultivars ($P<0.05$) (Fig. 7.9A and B). 1-NAA treatments also enhanced production of adventitious roots, even in the absence of waterlogging stress (Fig. 7.9A and B). Waterlogging also induced a significant ($P<0.05$) reduction of stomatal conductance (g_s) in both tolerant and sensitive cultivars (Fig. 7.9C). Both FHWL and 1-NAA treatments recover g_s to the similar values comparable with control plants. Total auxin content in the 2 cm region of shoot base (where most adventitious roots occur) was highest in FHWL treatment, following by WL treatment (significant at $P< 0.05$) (Fig. 7.9D).

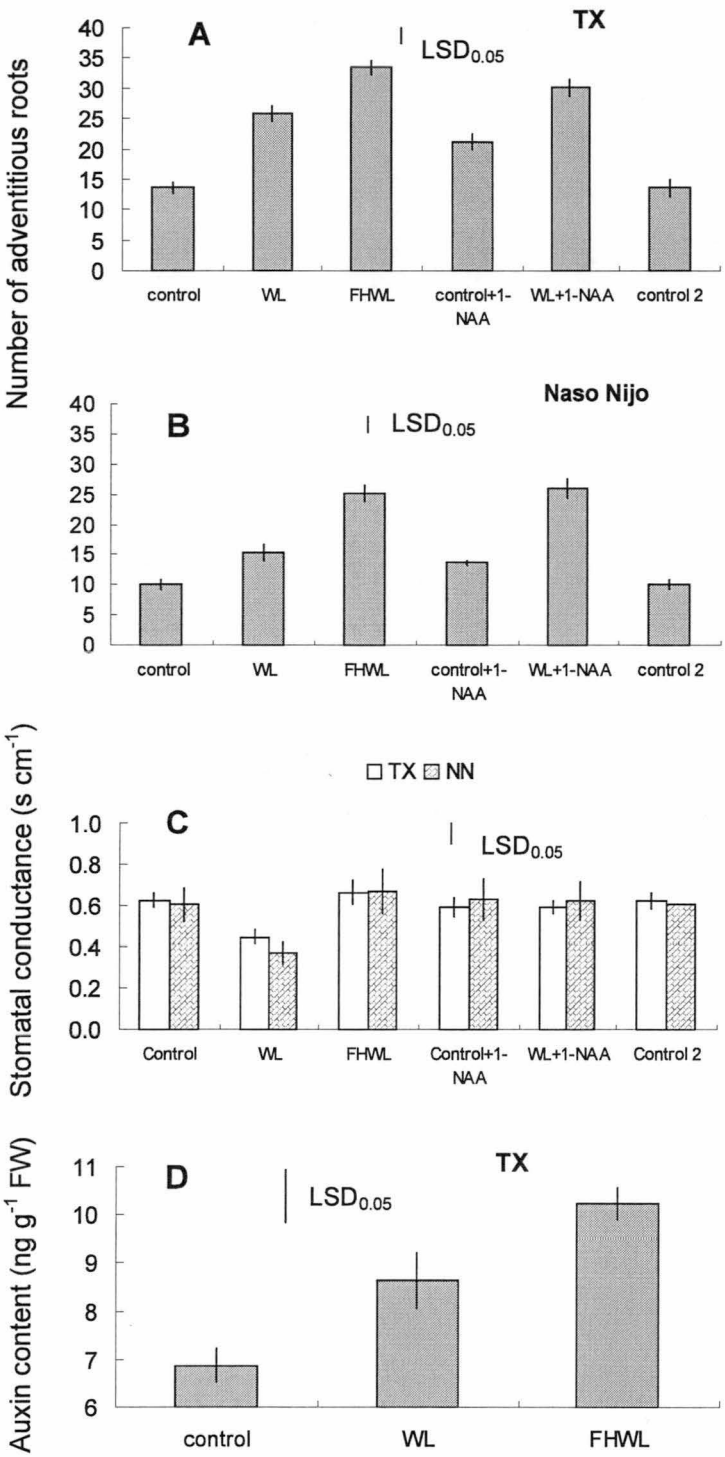

Fig. 7.9. Effects of 14 days treatment on the number of adventitious roots in TX9425 (A) and Naso Nijo (B); stomatal conductance in TX9425 and Naso Nijo (C); auxin content in 2 cm of shoot base regions in TX9425 (D). Error bars are SE (n=20 in A and B, n=10, 5 respectively for C, D)

It is known from literature that auxin distribution in plant tissues is rather heterogenic, synthesized mainly in shoot and root meristematic tissues and young leaves (Goldsmith 1977). It was found that the transport of auxin from the shoot to the root is hampered resulting in accumulation of auxin in a small area at the shoot-root junction due to high water levels under waterlogging conditions (Wample and Reid 1979). Accordingly, experiments were conducted, splitting the entire 2 cm stem region above shoot base into smaller 0.4 cm segments (5 in total). It was found that the length between 1.2 and 1.6 cm to the shoot-root junction had the highest auxin content, which was almost twice that other areas at both ends of the studied segment (Fig. 7.10).

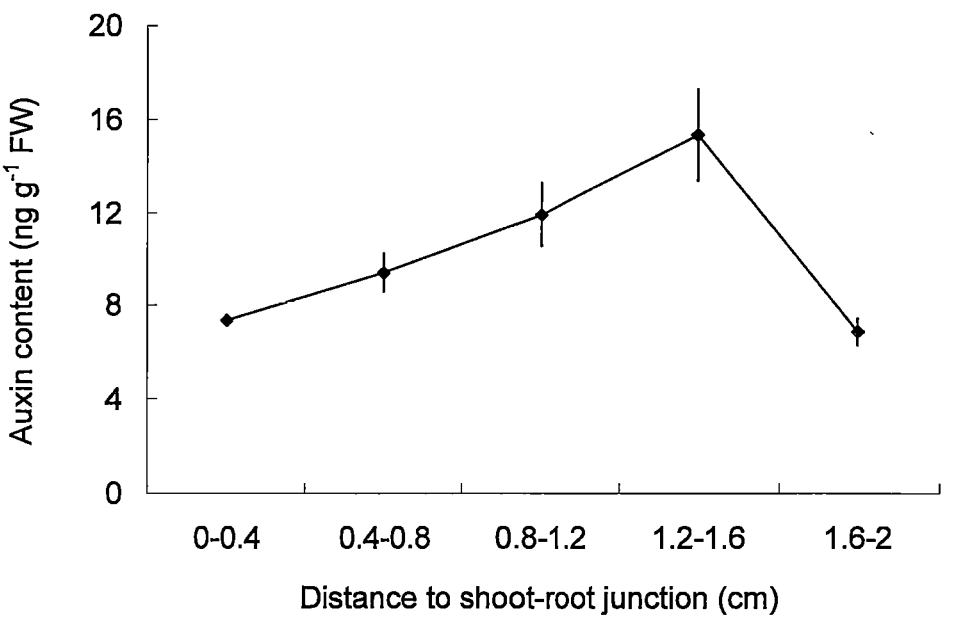

Fig. 7.10. Auxin profile in the barley shoot base as a function of distance from the root-shoot junction. Error bars are SE (n=3).

7.5. Discussion

Three major signalling systems coordinate root to shoot communication in plants: (1) hormonal signalling, (2) transport of assimilates and (3) electrical signalling.

The most rapid is electrical signalling. Many plants respond to various abiotic stimuli by activation and propagation of fast electrical signals, so-called action potentials (Fisahn *et al.* 2004). In higher plants, action potentials may be the information carriers in intercellular and intracellular communication in response to environmental changes and are the primary candidates for intercellular signaling in higher plants (Volkov *et al.* 2001). The action potentials are short pulses of electric (ionic) current which rapidly travel through the entire plant (Wildon *et al.* 1992). In situations in which rapid, systemic signaling responses are required, the generation of electrical signals via the sieve element and its companion cell (SE-CC) complexes could be potentially effective (Oparka and Turgeon 1999). At the cellular level in plants, electrical potentials exist across membranes, between cellular compartments, and within specific compartments. Ions such as K^+, Ca^{2+}, H^+, Na^+, and Cl^- represent electrolytic species involved in the establishment and modulation of electrical potentials (Findlay 2001, Volkov *et al.* 2001). Therefore, one of the possibilities was that propagating electrical signals, activated in response to foliar nutrient application, may travel basipetally and change nutrient uptake by roots, resulting in improved nutritional status in this organ (Fig. 7.7). However, immersing a leaf into nutrient solution did not cause any immediate responses of Ca^{2+}, K^+, H^+ ions within 50 mins of treatment (Fig. 7.6). Keeping in mind that the propagation rate for action potentials is in the range of several cm/s (Malone 1993, Stahlberg and Cosgrove 1997), it is unlikely that the electrical system is responsible for improved root nutritional status and production of adventitious roots.

My data here showed the general reduction of N, K and Ca upon waterlogging in the shoots except K content in TX (Fig. 7.3). A previous Chapter 5 and Pang *et al.* (2006) showed that oxygen deficiency caused an immediate reduction of K uptake in mature root zones in the waterlogging sensitive cultivar Naso Nijo, but this did not happen in the waterlogging tolerant cultivar TX9425. This might account for even the slight increase in K content in TX9425 after 2 weeks of waterlogging here, while K content was reduced in the other 3 cultivars. Sharma and Swarup (1989)

found that waterlogging caused significant reduction of Ca content in both shoot and root tissues of wheat, while Steiger and Feller (1994) found that Ca concentrations in the vegetative parts of wheat were hardly influenced after 38 days waterlogging, due to the low mobility of Ca. In this study, Ca in barley roots was hardly affected by waterlogging (Fig. 7.7). The improvement of shoot nutritional status (Fig. 7.3) might be the reason for observed reduction of leaf chlorosis, improved leaf photosynthetic characteristics and leaf biomass accumulation (Fig. 7.1 and 7.2). Consistent with this, Drew *et al.* (1979b) observed that the chlorosis and premature leaf senescence in flooded barley plants strongly resembles that caused by nitrogen deficiency.

In this study, foliar nutrient application not only increased the N and K contents in shoots, but also increased the contents of these nutrients in roots (Fig. 7.3 and 7.7). One possible explanation could be the translocation of foliar absorbed nutrients to the roots via the phloem. Extensive absorption and distribution of urea nitrogen throughout the plant within 24 hr have been shown by the use of N^{15}-labeled urea applied to sugar cane and tobacco (Wittwer and Teubner 1959). Muller *et al.* (1996) found that foliar uptake of N is not only supplementary, but can influence the N status of the whole plant. In spruce, foliar NO_2 uptake could account for up to 40% of NO_3^- uptake in short-term and up to 15% in long-term experiments of the whole-plant N-budget (Muller *et al.* 1996). In KNO_3 foliar sprayed *Ricinus communis* plants, one quarter of potassium was taken up by the shoots (Peuke *et al.* 1998). My data also showed that foliar nutrient application failed to improve Ca content in either shoots or roots (Fig. 7.3 and 7.7). This might be because Ca is an immobile element which is normally translocated solely in an acropetal or polar direction (Marschner 1995).

In addition to nutrient translocation from shoots to roots, the improvement of nutrient status in the roots could be also attributed to the production of adventitious roots. Adventitious roots typically grow from stem tissues rather than from the

regular root system of the plant. In this study, foliar application of IAA significantly increased the production of adventitious roots in both control and WL plants in both Naso Nijo and TX9425 ($P<0.05$) (Fig. 7.9A, B). Many studies have shown that significant production of adventitious roots caused by waterlogging is related to hormonal changes such as those of auxin and ethylene (McDonald and Visser 2003, Visser *et al.* 1996b, Visser *et al.* 1996c). Visser (1995) showed that application of either auxin or ethylene induces formation of adventitious roots in *Rumex palustris* and *Rumex thyrsiflorus* plants and that inhibition of auxin transport from the shoot to the rooting zone decreases the number of roots induced by flooding. In this study, two weeks of waterlogging stress caused significant accumulation of auxin in the shoot base in TX9425 compared with the control ($P<0.05$) (Fig. 7.9D). Auxin is known to be a major regulator of adventitious root formation (Blakesley 1994). Auxin transport is polar, more prominently downward from the shoot apices (Goldsmith 1977). Phillips (1964) proposed that the increased auxin concentration in the shoot of flooded sunflower plants could be due to either, (i) a cessation of auxin movement into the roots, or, (ii) inhibition of shoot-synthesized auxin oxidation in the root tissues, followed by reduced movement of auxin from shoot to root. Inhibition of ethylene synthesis in roots also led to a decline in root formation under flooded conditions (Visser *et al.* 1996c). Wample and Reid (1979) proposed that the transient rise in ethylene caused the buildup of auxin in the sunflower hypocotyl resulting in adventitious root formation. The peak in ethylene concentration preceded the build up of radioactive auxin in the flooding with aeration sunflower hypocotyls, which might play an important role in the production of adventitious roots (Wample and Reid 1979).

Foliar nutrient application on WL plants after 14 days in the present study significantly increased the production of adventitious roots compared with WL plants without extra foliar nutrient (Fig. 7.9A and B), which might be as a result of the accumulation of more IAA at the shoot base in FHWL plants than WL plants (Fig. 7.9D). This study showed that auxin was concentrated in a relatively narrow

region, with the highest content between 1.2cm and 1.6 cm from the shoot-root junction, close to the growing meristem in young barley plants.

It is unclear how improved nutrient status of the shoot can enhance auxin synthesis and/or transport towards the root. It has been reported that micronutrient zinc is required for the normal auxin biosynthesis. Zn is required for maintenance of auxin in an active state and the lack of Zn leads to excessive destruction (probably oxidation) of auxin (Skoog 1940). It was reported that waterlogging caused the significant reduction of Zn (as well as other nutrients) in lucerne plants (Smethurst *et al.* 2005). Therefore, it can be hypothesized that a supplement of Zn in the nutrient solution to the plant shoot in this study might play a role in the enhanced auxin synthesis and, hence, in larger amounts of auxin translocated towards roots. Specific details on the relationship between leaf nutrient status and auxin synthesis and transport remain an issue for future studies.

Stomatal opening is accompanied by the accumulation of potassium salts and sugar by the individual guard cells (Becker and Hedrich 2002). Foliar application of IAA alleviated the adverse effects of WL on stomatal conductance in leaves (Fig. 7.9C). Becker and Hedrich (2002) found that guard cells exhibit an auxin-dependent phenotype and the two guard cells that surround a stoma increase their volume and thereby open the stomatal pore in response to exogenous auxin. An auxin induced activation of the guard cell H^+-ATPases would hyperpolarize the membrane and thereby allow K^+ uptake into the guard cells through voltage dependent, inward-rectifying K^+ channels (Felle *et al.* 1991). In addition, the H^+ gradient would provide the driving force for the uptake of Cl^- and/or sugars via H^+-based symporters in the guard cell plasma membrane (Becker and Hedrich 2002).

In conclusion, results of this study suggest that the improvement of waterlogged barley growth by foliar nutrient spray could be related to both the larger amount of nutrients translocated from the shoot to the root via phloem, and to the auxin-induced increase in the formation of adventitious roots.

Chapter 8 General Discussion and Conclusions

8.1. Whole plant physiological responses and the prospects of Fv/Fm for screening

The growth response of 6 barley genotypes with different origins to waterlogging and subsequent recovery were studied. Both shoot and root growth were adversely affected by waterlogging. As waterlogging stress developed, chlorophyll content, net CO_2 assimilation and maximal photochemical efficiency of PSII (Fv/Fm) decreased significantly. The adverse effects in stressed plants were alleviated after 2 weeks of drainage in all genotypes. Among these 6 cultivars, it was found TX9425 showed the most waterlogging tolerance, while Naso Nijo was the most waterlogging susceptible.

Among all physiological parameters studied, the chlorophyll content and changes in photosynthetic parameters such as CO_2 assimilation rate and stomatal conductance were the most responsive to waterlogging stress and could potentially be used to screen barley lines for waterlogging tolerance. Their use for large-scale screening, when thousands of leaf samples need to be analysed is, however, offset by the significant amount of time required for analysis. Chlorophyll fluorescence measurements, however, are rather simple, rapid (only a few seconds per sample are required for measurements from dark-adapted leaves) and provide essentially the same information about the impact of waterlogging on photosynthetic machinery as CO_2 assimilation measurements or pigment analysis. Therefore, for a large-scale breeding program, chlorophyll fluorescence of dark-adapted samples (Fv/Fm values) is likely to be the most efficient parameter for screening plants for waterlogging tolerance. ,

8.2. Morphological and anatomical adaptations

One major problem in plant roots under waterlogging conditions is the lack of O_2, leading to the inhibition of energy-dependent processes. For a long-term survival, the exploitation of surface rooting and the development of aerenchyma in roots are necessary to facilitate gas diffusion from above-ground parts (Armstrong *et al.* 1991a). In order to understand the mechanism underlying waterlogging tolerance in barley, the morphological and anatomical differences between two barley cultivars contrasting in waterlogging tolerance were investigated. In both cultivars, most seminal roots died under waterlogging conditions, while adventitious roots were produced at the shoot base. More adventitious roots were found in the waterlogging tolerant cultivar than in the susceptible cultivar. In adventitious roots of waterlogged plants, a substantial amount of aerenchyma was formed in the bulk of the root cortex except at the apex, facilitating O_2 transport from the aboveground parts into submerged roots. No aerenchyma was present in well-drained plants. The aerenchyma percentage along the whole root in the waterlogging tolerant cultivar was much larger than in the sensitive cultivar. The percentage of stele and xylem to the cross-section in adventitious roots was significantly reduced compared with the well-drained plants. Taken together, it appears that targeting mechanisms of aerenchyma formation in barley has to be included in any breeding program aimed at improvement of waterlogging stress tolerance.

8.3. Electrophysiology and underlying ionic mechanisms

Membrane transport processes are known to be involved in virtually every aspect of plant life. Changes in plasma membrane potential and/or ion flux modulations are amongst the earliest cellular events ever measured in response to temperature, hormonal stimuli, elicitors, osmotic stress and mechanical stimulation in many organisms (Zimmermann *et al.* 1999). To understand the effects of waterlogging on nutrient acquisition and potential involvement of plasma membrane ion transporters

in waterlogging tolerance in barley, the net O_2 and ion fluxes from the root surface was concurrently measured using the non-invasive microelectrode MIFE system.

Using commercially purchased O_2 microelectrodes (Unisense, OX10 type, Aarhus, Denmark), O_2 fluxes around barley roots were measured. To my knowledge, so far there is only one laboratory in Italy (Mancuso and Boselli 2002) using a similar technique to directly measure O_2 flux in the plants. It was found in my work that oxygen deprivation caused the decline of O_2 uptake and an immediate and substantial effect on ion flux patterns in barley roots. These effects were different between waterlogging sensitive and tolerant cultivars. The O_2 uptake in the waterlogging tolerant cultivar remained much higher than in the waterlogging sensitive cultivar Naso Nijo in the root mature zone under hypoxia stress, while there was no significant difference in the root elongation zone between these two cultivars. This narrows the zone where "waterlogging tolerant genes" may be functionally expressed.

Hypoxia stress caused different ionic responses in mature and elongation zones of barley roots. In the mature zone, hypoxic treatment caused a very sharp decline in K^+ uptake in Naso Nijo, but did not reduce K^+ influx in the waterlogging tolerant TX9425 cultivar. In the elongation zone, onset of hypoxia enhanced K^+ uptake from roots of both cultivars. The ability of tolerant TX9425 to maintain relatively stable K+ uptake in the root mature zone might result from the higher O_2 uptake in this zone under hypoxia stress. Hypoxia also caused qualitatively different effects on the activity of plasma membrane ion transporters in mature and elongation zones. Pharmacological experiments suggested that hypoxia-induced K^+ flux responses are likely to be mediated by both KIR and NSCC channels in the elongation zone, while in the mature zone KOR channels are the key contributors. Altogether, these results suggest that efficient regulation of membrane transporters under anoxia is another potential avenue to improve plant waterlogging tolerance. This has to be kept in mind while targeting specific genes in the breeding programs.

Waterlogging is not only associated with O_2 depletion, but also causes a significant accumulation of toxic substances from the microbial reduction processes which have been widely reported in waterlogged soil. To my knowledge, plant tolerance to these secondary metabolites has never been considered as a useful trait in barley breeding programs. In this work, effects of several secondary metabolites (phenolic acids, monocarboxylic acids and Mn^{2+}) on nutrient (K^+, H^+ and Ca^{2+}) acquisition of barley roots were investigated. Phenolic and monocarboxylic acids both caused immediate and significant reduction of K^+ uptake, possibly via membrane depolarisation. Therefore, a plant's ability to restore membrane potential and retain K^+ in the cytosol is another potential source of improving waterlogging tolerance in barley. It also appears that Ca^{2+} signalling is likely to be involved in the response to phenolic acids. Accordingly, barley breeding programs should not only target the ability of plants to tolerate O_2 deprivation and/or increase O_2 supply to roots, but also consider their ability to withstand these toxic substances produced in waterlogging soil.

8.4. Alleviation of waterlogging by foliar nutrient application

On a practical side, a possibility of using foliar nutrient sprays to alleviate the adverse effects of waterlogging was investigated. Foliar application of full strength Hoagland solution significantly improved plant growth, reduced leaf chlorosis and increased chlorophyll content, photochemical efficiency of PSII, net CO_2 assimilation, and production of adventitious roots. Thus, foliar nutrient sprays may be recommended to be used under the field conditions to improve nutritional status and the overall performance of waterlogged plants. Interestingly, N and K content increased not only in shoots but also roots, suggesting the translocation of nutrient from the shoot to the root. This may be partially the reason for the greater production of adventitious roots in sprayed plants. Another contributing factor may be significantly higher amounts of auxin, accumulated in the shoot base of

waterlogged plants after foliar nutrient application. It remains to be tested if improving a plant's ability to synthesise and/or transport auxin is a useful trait for plant waterlogging tolerance.

8.5. Potential for the use of Chinese barley cultivars

This project is an integral part of GRDC-funded collaboration between Australia and China on barley genetic resources. To overcome the lack of waterlogging tolerant barley cultivars in Australia, some Chinese cultivars were imported and studied in this research. Among studied genotypes, the Chinese cultivar TX9425 was found to be the most waterlogging tolerant, with the least reduction of plant growth, chlorophyll content, chlorophyll fluorescence and photosynthetic parameters. Naso Nijo and Franklin were found to be the most susceptible to waterlogging. As the absolute biomass of TX9425 is relatively small, it cannot be used directly in farmers' fields. However, this cultivar could be used as a potential donor of waterlogging-tolerant genes in any breeding program on barley.

8.6. General conclusions

Overall, this research suggests that substantial genetic potential exists to improve waterlogging tolerance in barley. Measuring chlorophyll fluorescence of dark-adapted samples (Fv/Fm values) is recommended as an efficient screening tool for waterlogging tolerance in barley breeding program. Key features targeted by breeding should include both morphological (production of more adventitious roots and formation of larger aerenchyma area in adventitious roots) and physiological (high ability of O_2 uptake and K^+ acquisition in plant roots) traits, as well as ability of plants to withstand soil-borne phytotoxins. Foliar application of nutrients could be used in practice to alleviate the adverse effects of waterlogging.

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